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THE UNIVERSITY OF ALBERTA

STUDIES ON LATENT HERPES SIMPLEX VIRUS IN THE HUMAN NERVOUS SYSTEM

by

MARCIA ELLEN LEWIS

A THESIS

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The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies and Research, for acceptance, a
thesis entitled "Studies on Latent Herpes Simplex Virus in the Human
Nervous System" submitted by Marcia Ellen Lewis in partial fulfilment
of the requirements for the degree of Doctor of Philosophy in Medical
Sciences.

DEDICATION

To my parents, who have never failed to support and encourage my every endeavor. To them I give this gift of love and thanks.

To my uncle, Dr. Henry Kamin , who was probably responsible for my interest in science and whose career and personal integrity I can only hope to emulate.

ABSTRACT

Explantation and in vitro cultivation of human neurological tissue obtained from unselected cadavers has enabled the recovery of spontaneously expressed herpes simplex virus (HSV) isolates. Furthermore, techniques of in vitro reactivation of latent HSV have facilitated studies concerned with the biology of the virus in human cells and molecular aspects of HSV strains recovered from human hosts.

HSV was recovered from the trigeminal ganglia of 33 of a total of 82 (40.2%) individuals and from explant cultures of trigeminal nerve roots derived from 7 of 47 human cadavers. Isolates were identified as HSV-1 by restriction enzyme analysis. This finding indicated that in a minority of individuals clinically latent HSV-1 is not confined to autonomic and sensory ganglia. Recovery of HSV from human trigeminal nerve roots also suggested that latent virus may be present in cell types other than ganglionic neurons.

Trigeminal ganglion cultures derived from 36 individuals which failed to express HSV spontaneously were superinfected with genetic probes in the form of individual ts mutants of HSV-2 to detect the presence of putative genetic information able to complement or recombine with the input virus. Eight cultures from 6 individuals contained HSV-specific information which could be detected or rescued following superinfection. Restriction enzyme profiles of ts^{+} viruses recovered from the ganglia of two individuals following superinfection were identical to those of endogenous HSV-1 spontaneously released from parallel cultures. This finding suggested products of the superinfecting virus activated expression of whole genomes or that spontaneous virus expression occurred unrelated to the act of

superinfection.

Continuous incubation of explant cultures of human trigeminal ganglia in 10 μM acyclovir (ACV) prevented in vitro reactivation of HSV in 23/23 (100%) of cases. In cultures released from ACV, virus reactivation occurred in 3/18 cases. HSV was recovered from 9/20 (45%) of control cases. The above results illustrated that the continuous presence of ACV was able to inhibit HSV reactivation, though in several cases, without elimination of latent virus.

115 HSV isolates were recovered from explant cultures from the trigeminal ganglia of 20 cadavers, including one case in which virus was also recovered from the vagus ganglion and one in which virus was isolated from trigeminal nerve roots. All isolates had restriction enzyme profiles characteristic of HSV-1. In addition, HSV-1 isolates from 18 of 20 individuals could be differentiated from individual to individual with respect to the presence or absence of 16 specific restriction sites with 3 enzymes, though all isolates derived from a single host were identical. However, the DNAs of isolates from two individuals differed among themselves in the number and location of sites with at least two of three enzymes used. Recovery of non-identical strains of latent HSV-1 within single hosts suggested multiple strains arose by exogenous reinfection or by concurrent infection with more than one strain of HSV. Furthermore, the ability of an individual human host to harbor multiple strains of latent HSV within single or multiple ganglia indicated, if even in only a minority of individuals, that the immune response elicited against an initial herpesvirus infection may not be protective against subsequent exposures to strains of the same or different serotypes.

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LIST OF ABBREVIATIONS

ACV	acyclovir
BHK C113	baby hamster kidney cells clone 13
CNS	central nervous system
CPE	cytopathic effect
DNA	deoxyribonucleic acid
EMEM	Eagle's minimal essential medium
FBS	fetal bovine serum
G + C	guanine plus cytosire
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
mRNA	messenger ribonucleic acid
NPT	non-permissive temperature
PBS	phosphate buffered saline
PFU	plaque-forming unit
PNS	peripheral nervous system
PT	permissive temperature
RE	restriction enzyme
RNA	ribonucleic acid
SSC	0.15M NaCl, 0.015M sodium citrate
TK	thymidine kinase
tRNA	transfer ribonucleic acid
ts	temperature-sensitive
ts+	non-temperature-sensitive

CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

Introduction

Herpes simplex virus (HSV) is the prototype of a large group of viruses which have been isolated from a wide variety of animal species, including humans. Members of the group share many physical and morphological characteristics, yet differ in host range and biological properties. Five distinct human herpes viruses have been identified: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and varicella zoster virus (VZV) (Spear and Roizman, 1980).

Clinical syndromes associated with herpes simplex viruses include fever blisters or cold sores localized to the orofacial region, mucocutaneous lesions of the genitalia, encephalitis and severe generalized infections of the newborn or those undergoing immunosuppressive therapy (Nahmias and Roizman, 1973). Infections in animals or in a broad range of cultured cell types typically results in a lytic virus cycle with production of infectious progeny and a characteristic cytopathology. HSV-1 and HSV-2 can morphologically or biochemically transform cells in culture, but only if virion or genome integrity is disrupted by physical means or exclusion of certain viral genes (Spear and Roizman, 1980).

Characteristic of herpes simplex viruses are a linear double-stranded DNA genome which is approximately 100×10^{6} in molecular weight, a virion nucleocapsid containing 162 capsomeres and a membraneous envelope acquired as the nucleocapsid buds through

virus-specified glycoprotein containing regions of the host cell membrane (Spear and Roizman, 1980).

Considerable attention recently focused on herpes simplex virus is attributable to several factors. These include the epidemic of venereal disease caused by HSV, the putative association between HSV-2 and cervical neoplasia (Nahmias et al., 1973; Rawls et al., 1973), the propensity of these viruses to remain latent in nervous tissue throughout the lifespan of an individual (Stevens, 1975) and the search for an effective vaccine capable of preventing or attenuating the diverse range of clinical infections caused by HSV.

Aspects of the HSV replicative cycle and assembly, synthesis of proteins and RNA in infected cells, the replication of HSV DNA and cellular transformation by HSV have been extensively reviewed elsewhere (Spear and Roizman, 1980; Roizman, 1979) and will not be discussed here. The following review will concentrate on characteristics of the virion, HSV DNA, the genetics of HSV and aspects of latent infections in animals and man.

Structure of Virion

Morphology and size

The virions of herpesviruses contain an electron-opaque core, an icosahedral capsid surrounding the core, the tegument surrounding the capsid which is made of an electron-dense amorphous material and an envelope whose outer surface has spike-like projections (Roizman and Furlong, 1974).

Viral DNA is present in the core and is responsible for its electron-opacity. Agents which destain DNA or degrade it, such as DNAse reduce electron density (Epstein, 1962a; Furlong et al., 1972). The capsid consists of 162 capsomers and is approximately 100 nm in diameter (Wildy et al., 1960). The tegument consisting of material located between the capsid and the envelope has been shown to vary in amount from virion to virion even within the same cell (Roizman and Furlong, 1974; Wildy et al., 1960). The outer envelope of the virion is composed of a lipid bilayer and associated proteins and has a typical laminar appearance electron microscopically (Epstein, 1962b). The viral membrane is permeable (Watson and Wildy, 1963), sensitive to lipid solvents and detergents (Hamparian et al., 1963; Spring and Roizman, 1968; Kaplan and Ben-Porat, 1970; Olshevksy and Becker, 1970; Spear and Roizman, 1972) and is derived from altered regions of the host cell membrane (Falke et al., 1959; Morgan et al., 1959; Armstrong et al., 1961).

HSV DNA

The genome of herpes simplex virus (HSV) types 1 and 2 consists of a linear double stranded DNA molecule of approximately 100×10^6 daltons (Becker et al., 1968; Kieff et al., 1971; Wilkie, 1973). HSV-1 and HSV-2 DNAs show about 50% sequence homology (Kieff et al., 1972) and have a base composition of 67 and 69 G+C mole % respectively (Kieff et al., 1971). A single viral particle or isolated DNA molecule is sufficient to initiate productive infection (Sheldrick et al., 1973; Lando and Rhyiner, 1969). The DNA molecule is unsual in that it often contains some alkali-labile interruptions in the linear sequence of either or both single strands (Kieff et al., 1971; Frenkel and Roizman, 1972; Wilkie, 1973). The sequence arrangement of HSV DNA can be described as follows: (1) HSV DNA consists of two covalently-linked components designated L and S comprising 82% and 18%, respectively, of the viral DNA. Each component consists of unique sequences (U_L and U_S) bracketed by inverted repeats. This model was based on the finding that intact single strands, upon self-annealing, form two single-stranded loops of unequal size bridged by double-stranded DNA (Sheldrick and Berthelot, 1974). (2) The reiterated sequences bracketing the L component have been designated as "ab" and "b'a'" and each comprises 6% of the total DNA; the reiterated sequences flanking the S component have been designated as "a'c'" and "ca" and each contains 4.3% of sequences of the DNA which differs in average base composition from the "ab" and "b'a'" sequences. These conclusions were based on measurements of the double-strand regions formed by self-annealing of intact strands and on partial denaturation studies of intact molecules (Wadsworth et al., 1975). (3) HSV DNA can be circularized after digestion with a

processive exonuclease (Grafstrom et al., 1975; Sheldrick and Berthelot, 1974), but the size of the cohesive ends, designated as the "a" sequence is uncertain (Wadsworth et al., 1975).

Sheldrick and Berthelot (1974) suggested that because each unique region in the HSV genome is flanked by terminal regions which are inverted internally, either terminal repeat can pair with its internal complementary sequence, leading to inversion of the L or S components. Subsequently it was established that the L and S components can indeed invert relative to each other and that DNA extracted from wild-type virions consists of four equimolar populations differing solely in the orientation of L and S components. These populations have been designated prototype (P), inversion of the S component (I_S), inversion of the L component (I_L), and inversion of both S and L components (I_{SL}) (Roizman et al., 1974; Hayward et al., 1975b). The evidence for the existence of four populations is based on identification of terminal and junction restriction fragments (Roizman et al., 1974; Hayward et al., 1975b Clements et al., 1976) and on partial denaturation profiles (Hayward et al., 1975b; Delius and Clements, 1976).

A consequence of the inversions of L and S components, which also led to the discovery of HSV genomic isomerization, is that for a restriction enzyme which cleaves HSV DNA outside the inverted repeats, three types of fragments will be generated: (1) 1.0 molar fragments produced from the unique regions of the DNA which are unaffected by an inversion of these regions, (2) four 0.5 molar fragments which are generated from the ends of the molecules, and (3) four 0.25 molar fragments each of which spans the two internal repeats. Enzymes which cleave within inverted repeat regions as well as in unique regions

generate only 1.0 molar and 0.5 molar fragments (Clements et al., 1976).

Recent work has focused on the structure and role of the genomic termini in inversion, circularization and replication of HSV DNA. Previous studies have shown the size of the terminal and internally inverted "a" sequence in different strains of HSV-1 to be 400 to 1600 base pairs (bp) by electron microscopy (Grafstrom et al., 1975) and 265 bp by restriction endonuclease mapping (Wadsworth et al., 1975). The above findings attributed size heterogeneity in the joint and terminal regions to variations in the number of copies of the "a" sequence at the L-S junction and at the L terminus. Nucleotide sequence data has also revealed the existence of three separate sets of direct repeats which vary in copy number within the "a" sequence itself (Davision and Wilkie, 1981; Mocarski and Roizman, 1981). Because adjacent "a" sequences share an intervening direct repeat, it was suggested that amplification or reduction in the number of "a" sequences can occur by recombination through this direct repeat (Mocarski and Roizman, 1982). Furthermore, it was shown that the free termini of the L and S components form one complete direct repeat sequence and therefore that linear unit-length molecules are generated by cleavage of head-to-head concatameric structures within the direct repeat (Mocarski and Roizman, 1982).

Functionally, the "a" sequence has been shown to mediate intramolecular inversion; thus the insertion of DNA fragment spanning the joint and containing an "a" sequence into the middle of U_L causes new inversions to occur (Mocarski et al., 1980; Mocarski and Roizman, 1981, 1982; Smiley et al., 1981).

Restriction endonuclease maps and variability of HSV DNAs

Restriction endonuclease maps of HSV-1 and HSV-2 DNAs have been constructed for a variety of enzymes (Frenkel et al., 1976; Wilkie, 1976; Jones et al., 1977; Skare and Summers, 1977; Cortini and Wilkie, 1978) and reveal differences between HSV-1 and HSV-2 DNAs (intertypic variability) (Wilkie, 1976; Skare and Summers, 1977; Cortini and Wilkie, 1978) as well as among HSV-1 or HSV-2 DNAs (intratypic variability) (Hayward et al., 1975a; Skare et al., 1975).

Subsequent analyses of viral DNA (Buchman et al., 1978; Hammer et al., 1981, unpublished results cited in Roizman, 1979) disclosed that no epidemiologically unrelated isolates of HSV-1 were identical. The major criteria used to establish genetic non-identity were differnces in the presence or absence of restriction enzyme cleavage sites and the existence of occasional deletions. These workers reported analysis of more than 80 isolates with six restriction enzymes revealed 19 out of 60 sites that could be present or absent. Variability at each site appeared to be independent of others. This observation was substantiated only in part by Chaney et al. (1983), since it was reported that simultaneous consideration of two or more variable sites among 29 HSV-1 facial and genital isolates disclosed some which were correlated.

Genomic stability of HSV DNAs in vivo and in vitro as measured by the consistency in the number and location of restriction enzyme sites has been well documented (Buchman et al., 1980; Roizman, 1979). Extensive in vitro passaging of virus strains, re-isolation of HSV-1 from the same patient over a 12 year period, and isolation of virus from patients with recurrent infection and from epidemiologically related

individuals have all failed to reveal a single instance of spontaneous loss or acquisition of a restriction site (Buchman et al., 1980).

Variability among HSV-2 DNAs has been reported to be less extensive than that observed in HSV-1 (Buchman et al., 1979; Chaney et al., 1983), though this finding may be attributable to both the enzymes utilized in these studies and the fact that less data is available for HSV-2 than for HSV-1.

Variability in restriction enzyme profiles of HSV DNAs has been applied to problems of epidemiology, e.g. to trace the spread of virus from one individual to another (Buchman et al., 1978; Linnemann et al., 1978) and to demonstrate that a temporal cluster of HSV encephalitis was a random event (Hammer et al., 1980). Of particular interest was the use of this technology to establish that individuals can become overtly infected with more than one strain of HSV within a serotype, either at the same or different sites (Buchman et al., 1979; Whitley et al., 1982). Restriction enzyme analysis has also shown that individuals can become concomitantly and recurrently infected with both HSV-1 and HSV-2 at the same genital site (Fife et al., 1983).

In accordance with the findings that in the majority of cases, clinical HSV isolates from unrelated hosts are nonidentical (Buchman et al., 1980), restriction endonuclease analysis of DNAs of latent HSV-1 recovered from human cadavers has shown that isolates from the ganglia of different individuals can be distinguished from one another. However, isolates from different ganglia or multiple isolates derived from a single ganglion within the same individual are indistinguishable (Lonsdale et al., 1979). Cloning of single virus isolates from a limited number of human sensory ganglia has also failed to reveal the

existence of more than one strain of virus within an individual (Lonsdale et al., 1980).

In the above instances, variation in the number and location of restriction sites has been used to establish genetic dissimilarities between virus isolates, however variation in the electrophoretic mobility of DNA fragments arising from terminal and internal repetitive regions of the HSV genome has been widely reported (Buchman et al., 1980; Locker and Frenkel, 1979; Lonsdale et al., 1979, 1980; Roizman and Tognon, 1982).

Roizman and co-workers (Roizman, 1979; Buchman et al., 1980) have ascribed HSV variability to the mode of transmission and epidemiology of the virus. Because of the propensity of HSV to remain latent within the human host and upon reactivation to be transmitted to new contacts, it was hypothesized that HSV strains are not displaced in the population. Therefore, nonlethal mutations are thought to accumulate in the population and become stabilized (Buchman et al., 1980).

Genetics of herpes simplex viruses

The isolation and characterization of nonspecialized conditional lethal temperature-sensitive (ts) mutants of both HSV-1 and HSV-2 have been reported by several laboratories (Subak-Sharpe, 1969; Brown et al., 1973; Manservigi, 1974; Schaffer et al., 1970, 1973; Timbury, 1971; Esparza et al., 1974; Halliburton and Timbury, 1973, 1976). By complementation analysis, a wide range of ts mutants of HSV-1 and HSV-2 has been assigned to functionally different complementation groups (Brown et al., 1973; Schaffer et al., 1973; Timbury, 1971; Halliburton and Timbury, 1976; Koment and Rapp, 1975). Complementation analysis of ts mutants derived in 10 laboratories has led to the identification of 29 and 20 essential cistrons of HSV-1 and HSV-2, respectively (Schaffer et al., 1978; Timbury et al., 1976).

Recombination among ts mutants of herpesviruses was first demonstrated by Subak-Sharpe (1969). The construction of linear linkage maps for HSV-1 and HSV-2 based on recombination between ts mutants has also been reported (Brown et al., 1973; Brown and Jamieson, 1978; Schaffer et al., 1974; Benyesh-Melnick et al., 1974; Timbury and Hay, 1975; Timbury and Calder, 1976). However, because of the inversion of the L and S components of HSV DNA relative to one another, no direct comparison can be made between the order of mutants on the linkage map and their order on the genomic DNA (Schaffer, 1981).

In order to map ts mutants, viral proteins and functional markers on the HSV genome, several physical mapping procedures have been developed. Many of these technologies stemmed from experiments of Kieff et al. (1972) and Wilkie et al. (1978a) which investigated the overall DNA homology as well as specific distribution of homologous sequences

shared by HSV-1 and HSV-2. Studies of Wilkie et al. (1978a) using restriction endonuclease fragments indicated that homologous regions in the DNA of HSV-1 and HSV-2 are distributed widely throughout the unique long and unique short regions of the genome, thus allowing the alignment of physical maps for these regions. Since the restriction enzyme maps for HSV-1 and HSV-2 show numerous differences, it has been possible to determine the parental origin of DNA sequences of intertypic recombinants and map the crossover sites. Both intertypic and intratypic marker rescue have been used to map ts mutants of HSV, the DNA polymerase locus and many HSV-induced polypeptides (Stow and Wilkie, 1978; Wilkie et al., 1978a; Chartrand et al., 1979, 1980; Marsden et al., 1978; Morse et al., 1978; Preston et al., 1978; Ruyechan et al., 1979). In addition, marker transfer techniques have been used to determine the physical location of several genes, including that of virus-specified thymidine kinase (TK, Knipe et al., 1979; Pellicer et al., 1978; Wigler et al., 1977). Mapping of genes has also been accomplished by in vitro translation of mapped mRNA (Anderson et al., 1981; Frink et al., 1983; Preston, 1977a,b) and by microinjection and expression of mRNA into Xenopus oocytes (Cordingley and Preston, 1981; McKnight and Gavis, 1980; Preston and Cordingley, 1982).

It has been shown that sequences encoding immediate early transcripts and polypeptides map predominantly in the reiterated sequences bracketing U_L and U_S whereas sequences specifying early and late polypeptides and glycoproteins map mostly in unique sequences. (Clements et al., 1977; Easton and Clements, 1980; Marsden et al., 1978; Morse et al., 1978; Preston et al., 1978; Ruyechan et al., 1979). As inferred from DNA homology studies, the genes encoding the majority of

HSV-1 and HSV-2 polypeptides are colinear. One exception is the glycoprotein C (gC) gene, the HSV-2 counterpart mapping to the right of HSV-2 gC (Ruyechan et al., 1979).

Functional markers which have been mapped in HSV-1 and/or HSV-2 include four loci governing syncytia formation, the locus Cr which effects accumulation of gC (Ruyechan et al., 1979), TK (Halliburton et al., 1980; McDougall et al., 1980; Reyes et al., 1982; Wigler et al., 1977) and loci which specify resistance to phosphonoacetic acid (PAA^r) and acycloguanosine (ACG^r) which are associated with the viral DNA polymerase (Coen and Schaffer, 1980; Chartrand et al., 1979; Crumpacker et al., 1980; Knipe et al., 1979a). HSV-2 functional markers which have been mapped include the nuc$^-$ locus which regulates expression of alkaline exonuclease activity (Moss et al., 1979) and the locus responsible for shutoff of host cell protein synthesis (Morse et al., 1978).

Ts mutations which have been mapped include those derived from HSV-1 strains KOS (Parris et al., 1980; Chartrand et al., 1980; Dixon and Schaffer, 1980; Chu et al., 1979; Morse et al., 1977), 17 (Chartrand et al., 1979; Stow et al., 1978; Stow and Wilkie, 1978), HFEM (Ruyechan et al., 1979; Knipe et al., 1978), 13 (Knipe et al., 1979b) and HSV-2 HG52 (Chartrand et al., 1980, 1981).

Summaries of the map locations of genes specifying viral polypeptides and functions of HSV-1 and HSV-2 in lytic infections, and positions of various mutations are available in reviews by Schaffer (1981) and Spear and Roizman (1980).

Recently, site specific mutagenesis of restriction endonuclease fragments has enabled the production and characterization of several

temperature-sensitive mutants of HSV-1. These mutations enabled the identification of three new complementation groups in HSV-1 (Chu et al., 1979; Conley et al., 1981).

Additional features of well studied ts mutants such as those which map in the DNA polymerase locus and the gene specifying the immediate-early polypeptide $V_{mw}175$ (ICP 4) are discussed in the above mentioned review articles (Schaffer, 1981; Spear and Roizman, 1980).

Latency of herpesviruses

Introduction and historical review

One of the hallmarks of herpesviruses, as exemplied by HSV, is the propensity of the virus to establish a persistent state following initial overt infection. Though the involvement of nervous tissue in HSV latent infection has been inferred for many years, only recently has direct evidence been obtained to support the association between specific neural structures and residence of the HSV genome (Stevens, 1975, 1978).

At the turn of the 19th century, Howard (1903, 1905) noted the concurrence of herpetic lesions, trigeminal ganglionitis and pneumonitis. In 1905 Cushing, in the course of treating cases of trigeminal neuralgia by removal of corresponding ganglia, documented the development of herpetic lesions in dermatomes subserved by the contralateral, but not ipsilateral nerves. This finding suggested an intact trigeminal tract was requisite to the development of herpetic disease. In 1923, it was shown that in animals, corneal inoculation of HSV resulted in the appearance of gross and microscopic lesions in the trigeminal tract of the pons and medulla on the side ipsilateral to injection (Goodpasture and Teague, 1923). It was concluded infection ascended to the central nervous system (CNS) via the trigeminal nerve. Additional experiments by Goodpasture (1925) established the relationship between peripheral infection and CNS disease and introduced the hypothesis HSV was transmitted through nerve trunks. Several years later, Goodpasture (1929) suggested the possibility that HSV resides in a latent state within human nervous tissues, perhaps primarily within nerve cells of the ganglion.

Andrewes and Carmichael (1930) demonstrated 3 of 4 individuals sampled randomly possessed measureable and significant titers of neutralizing antibody to HSV as did individuals with a history of recurrent herpetic lesions. Later Dodd et al. (1938) and Burnet and Williams (1939) showed that primary infection with HSV was followed by the appearance of specific neutralizing antibody. The induction of an immune response following HSV infection was thus established, though the mechanism responsible for recurrent overt infection remained unresolved.

In the early 1950's Carton and Kilborn (1952) and Carton (1953) demonstrated that in patients undergoing surgical treatment for trigeminal neuralgia, transection of the proximal trigeminal nerve root was followed by the appearance of herpetic lesions at the periphery. They also showed lesions did not appear if the nerve was sectioned distal to the ganglion. At this time, it was concluded these manipulations resulted in activation of virus from the skin. However in 1964, Paine, referring to the above studies (Carton and Kilborn, 1952; Carton, 1953), stated manipulations of the trigeminal nerve root result in the appearance of peripheral herpetic lesions only if the ganglion has not been destroyed and peripheral divisions remain intact.

On the basis of the above clinical data Stevens (1975) presented the following general hypothesis to explain recurrent herpetic disease: primary infection results in viral replication in epithelial cells of the skin or mucous membrane with subsequent invasion of superficial nerve endings. Virus then migrates intra-axonally in sensory nerves to the corresponding sensory ganglion where a latent infection is established, most probably in neurons. Following "activation", virus travels centrifugally from the neuronal soma through nerve axons to the

periphery resulting in overt infection of epithelial cells.

Animal model systems of HSV latency

The initial and best studies animal model system which demonstrated sensory ganglia are the primary source of latent and reactivable HSV was the one developed by Stevens and co-workers (Stevens and Cook, 1971, 1973a, 1973b). In this system, viral inoculation into the rear footpads of mice was shown to be followed by centripetal movement of the infection through the peripheral and central nervous system to the brain. All mice that survived this infection harbored HSV in a reactivable form in the ipsilateral lumbosacral spinal ganglia.

At this time a working definition of latent HSV was developed. Thus latent virus could not be recovered from tissues by direct assay, such as by productive infection of indicator cells, detection of viral antigens or visualization of virus particles by electron microscopy, but could be recovered by explantation or co-cultivation techniques. Following establishment and maintainance of in vitro cultures virus particles were detectable in culture fluids. In addition, it was shown that latent infection of ganglia probably persisted throughout the lifetime of the animal.

Selective association of virus within ganglion cells was inferred since the aforementioned techniques failed to reveal the presence of HSV in peripheral tissues, sciatic nerve or central nervous system of animals which harbored virus in the ganglion. In addition, Cook and Stevens (1976) concluded HSV has a predilection for a variety of neural tissues especially the sensory ganglion cells, since after intravenous inoculation of animals, virus was detected commonly in dorsal root ganglia. In the same animals virus was recovered less frequently in

portions of the brain and spinal cord, but never from the lymphatic system, spleen, kidney, lung or liver.

Similar experimental protocols have resulted in the recovery of latent HSV from the trigeminal ganglia of rabbits (Nesburn et al., 1972; Stevens et al., 1972) and mice (Knotts et al., 1974; Walz et al., 1974) following corneal inoculation; from the trigeminal, cervical and lumbosacral spinal ganglia of mice after inoculation of the lip, ear, and vagina, respectively (Hill et al., 1975; Stevens, 1975; Walz et al., 1974); and in lumbosacral spinal ganglia of guinea pigs following inoculation of virus into the rear footpads (Scriba, 1975).

Reactivation of latent infection following defined manipulation has been achieved in several model systems. Walz et al. (1974) demonstrated reappearance of overt HSV infection within the sensory ganglia, but not in the footpad of mice following sciatic neurectomy. Nesburn and Green (1976) showed that in rabbits latently infected in the trigeminal ganglia, mechanical stimulation of the ganglia resulted in shedding of virus in the tear film in the majority of animals. Hill and associates (1978) have provoked virus reactivation with reappearance of overt infection at the site of peripheral inoculation, by the minor trauma of stripping the mouse ear skin with cellophane tape. Epinephrine iontophoresis to the rabbit cornea has also been shown to induce ocular shedding of HSV-1 in latently infected rabbits (Kwon et al., 1981). Stevens et al. (1975) were able to reactivate a latent sacrosciatic ganglionic infection by intratracheal instillation of Diplococcus pneumoniae. Following irradiation or cyclophosphamide treatment of latently infected immunocompetent mice, Openshaw et al. (1979) have demonstrated virus reactivation in the presence of antibody.

To determine viral functions essential for latent infections, Tenser and co-workers (Tenser et al., 1979) found that TK-negative mutants of HSV established latent infection in the trigeminal ganglia of both guinea pigs and mice with a lower frequency than that of TK-positive virus, suggesting that expression of this enzyme is necessary for latency. In a later study, Watson et al. (1980) identified six temperature-sensitive mutants of HSV-1 which produce latent infections in mice with reduced efficiency. One of these mutants (tsK) has a lesion in the immediate-early polypeptide V_{mw} 175 and is blocked at the immediate-early stage of infection (Preston, 1979; Watson and Clements, 1980). Thus, expression of at least one immediate-early and perhaps additional polypeptides expressed at later stages of infection may be requisite to establishment and maintenance of latent virus.

Though residency of latent HSV has previously been associated with specific neural structures (Stevens et al., 1975) there have been several reports implicating peripheral cutaneous sites with viral latency. Scriba (1977) has demonstrated that HSV-2 is recovered preferentially, and HSV-1, exclusively, from footpad (site of primary inoculation) of latently infected guinea pigs. More recently Hill et al. (1980) have recovered HSV from the ears of clinically normal latently infected mice. Similarly, Al-Saadi et al. (1983) have shown that latent infection in the mouse can be established by wild-type and ts mutants of HSV-2 in both the footpads and dorsal root ganglia, independently. The above results suggested virus can be present in the skin without associated disease. Whether such findings are indicative of microfoci of overt infection at the periphery related to reactivation

from associated ganglia, or the result of reactivation of latent virus from cells of cutaneous origin remains to be determined. Such findings call into question possible differences between "true" latency and low level persistent infections.

The use of animal model systems has also enabled the detection and in some cases, the recovery of latent HSV from tissues of the central nervous system. The putative presence of HSV within the CNS was suggested by the early work of Goodpasture and Teague (1923) discussed previously. More recently, Kristensson (1970) and Kristensson et al. (1979) described CNS lesions in mice after intradermal and intramuscular injection of HSV. Following corneal inoculation in rabbits, Townsend and Baringer (1978) reported the presence of trigeminal root entry zone lesions though adjacent peripheral nervous system tissue was spared. Knotts et al. (1973) showed HSV can induce a latent infection in the brainstems of rabbits and in the spinal cords of mice which had recovered from acute infection of the CNS. Cook and Stevens (1976) reported the presence of latent HSV in anterior and posterior portions of the brain of mice following generalized infection. Latent infections in the brains of mice were also established with temperature-sensitive mutants of HSV-1 (Lofgren et al., 1977).

In 1980 Cabrera et al., detected HSV DNA sequences by DNA reassociation kinetics in 30% of brains of mice harboring latent HSV in their trigeminal ganglia. HSV was recovered from only 5% of brains by explantation or co-cultivation. It was concluded that virus is maintained in brain tissue in a state which cannot always be reactivated by explantation techniques. The recent studies of Rock and Fraser (1983) have demonstrated that HSV-1 DNA can be detected in the CNS

tissue of latently infected mice by Southern blot hybridization technology. HSV DNA was found in approximately 50% of whole mouse brains examined, but was detected in the brainstem of all animals when defined regions of the brain were examined individually.

Latent HSV in human tissue

Clinically latent HSV type 1 (HSV-1) was first isolated from human trigeminal ganglia taken at autopsy by Bastian et al. (1972) by co-cultivating ganglia fragments with cells permissive for HSV replication. Subsequently, Plummer (1973) rescued HSV-1 from the trigeminal ganglia of 4 of 10 individuals and Baringer (1974) isolated HSV type 2 (HSV-2) from the sacral ganglia of 4 of 26 cadavers by co-cultivation techniques. Baringer and Swoveland (1973) successfully recovered HSV from the trigeminal ganglia of six of seven individuals by in vitro cultivation of ganglia fragments without co-cultivation with other cells.

HSV was isolated from the trigeminal ganglia of 12 of 20 randomly selected cadavers by Warren et al. (1977). Fragments of each ganglion were cultivated in vitro as explantation monolayers and were also co-cultivated with WI-38 and African green monkey kidney cells. HSV was expressed from explantation monolayers derived from 11 of 12 individuals but only from 5 of 12 individuals when co-cultivation techniques were employed. In a later study (Warren et al., 1978) HSV was isolated from trigeminal ganglia in explant monolayers from three of nine individuals; co-cultivation of ganglia with other cell types did not facilitate recovery of HSV. In addition latent HSV was recovered from 2 superior cervical ganglia and 1 vagus ganglion of 9 human cadavers. This observation indicated latent virus may reside in autonomic or sensory

ganglia other than the trigeminal and therefore may be implicated in the pathogenesis of recurrent disease at additional anatomical sites.

Forghani et al. (1977) reported the isolation of HSV-1 from the trigeminal ganglia of 44 out of 90 individuals, from the thoracic ganglia of 2 out of 25 and from the sacral ganglia of 21 out of 68 cases; HSV-2 was recovered from the sacral ganglia of 8 out of 68 individuals. In all cases where HSV was isolated from the ganglia and serum was available, homologous, type-specific antibody was demonstrable by radioimmunoassay (RIA). Where HSV-1 was isolated from the trigeminal ganglia and HSV-2 from sacral ganglia of the same individual, antibody to both virus types was present in the sera. This finding indicated simultaneous latent infections with each of two virus can occur, and that antibody is produced to each virus independently. In 8 of 10 cases where virus isolation attempts were negative, antibody to HSV-1 or HSV-2 or both was present. It was concluded that RIA may be a more sensitive method than in vitro culture techniques for detecting the presence of latent virus, or that latent HSV may reside in additional, unsampled sites in the body.

Recently latent HSV was reactivated from uterosacral ligaments from 5 of 12 cases of cervical carcinoma, and from one of 11 control cases. It was concluded that latent HSV resides in pelvic tissue distinct from the sacral ganglia and may serve as a source of virus causing recurrent infection and perhaps carcinoma of the cervix (Kitchener et al., 1982).

Trigeminal ganglia explant monolayers which do not spontaneously express HSV have been shown to contain genetic information, possibly in the form of uninducible or defective viral genomes, capable of complementing and recombining with superinfecting temperature-sensitive

mutants of HSV-1 (Brown et., 1979).

In 1979, Sequiera et al. demonstrated the presence of HSV genomes by in situ hybridization in the brains from 3 of 4 elderly patients who had died with chronic psychiatric illness and neuropathological changes but not in brain tissue from 2 patients who had acute psychotic episodes and minimal abnormal histology.

In situ hybridization technology has also revealed the presence of HSV-specific mRNA in 0.4-8.0% of human paravertebral ganglion cells (Galloway et al., 1979). In a latter study, it was reported that in ganglia from 14 of 40 individuals, HSV-2 transcripts from the left-hand 30% of the viral genome were detected (Galloway et al., 1982). However, RNA homologous to other sequences in the L component was present less frequently and no RNA from the S component was detected. These workers concluded specific and limited transcription of the HSV genome occurs in latently infected human ganglion cells.

Fraser et al. (1981) reported HSV-1 DNA sequences were found in 6 of 11 human brain DNA samples by Southern blot analysis. Different anatomical sites of the brain were assayed for the presence of HSV DNA. It was found no unique site for HSV DNA exists within the human CNS, nor is there a correlation between neurological disease (multiple sclerosis) and the presence of HSV-1.

Cell type involved in latent infections

In the early 1970's, Stevens and co-workers conducted a series of experiments which strongly suggested latent HSV is harbored exclusively in the neuron cell bodies of sensory ganglia (Cook et al., 1974). Their conclusions were based on the following observations: (1) Sciatic nerve trunks do not harbor latent virus. Since the neuronal soma is the only

cell type present in the ganglia, but not in the nerve trunk, the soma is the presumed site of virus latency. (2) If latently infected murine ganglia were transferred into the peritoneal cavity of uninfected syngenic mice, infectious viral progeny were produced. Immuno-fluorescent methods utilizing specific viral antigens and electron microscopic observations indicated the neuron was the first cell in which viral products or particles were detected. (3) Autoradiographic techniques illustrated neurons were the first cells in which viral DNA replication could be detected. When ^{3}H-thymidine pulses were followed by chases with unlabeled thymidine, radioactivity was found to be localized in satellite cells surrounding degenerated neurons. Additionally, the DNA in "reactivitating" neurons was identified as HSV-specific by in situ hybridization. The last observation has been documented by another laboratory (zur Hausen and Schulte-Holthausen, 1975).

More recently, McLennan and Darby (1980) confirmed the earlier conclusions of Cook et al. (1974) and provided evidence which suggested activation of latent virus in a neuron may lead to death of that cell. Latent infections were established with a ts mutant of HSV-1 unable to replicate at the body temperature of the murine host. When latently infected ganglia were cultured in vitro at the non-permissive temperature, immunofluorescence assays coupled with histological examination indicated viral products were associated with neurons. HSV antigens were also demonstrable in neurons following in vivo re-activation of latent virus by neurosurgery. Finally, when ganglia were excised several weeks after neurosurgery, there was a dramatic decrease in the number of ganglia from which latent virus could be recovered by

in vitro culture and in the quantity of virus produced. These data indicated a significant reduction in the number latent foci presumably due to the destruction of cells by virus reactivation. Despite evidence which implicated the neuron as the site of HSV latency, these workers were unable to rigorously exclude the possibility that other cell types may be involved in latent interactions.

Kennedy et al. (1983) reported the in vitro reactivation of HSV from dissociated dorsal root ganglia of mice. Double immunofluorescence labeling techniques showed cytopathic effect and HSV antigens arose first in neuron cells. These findings are consistent with those cited above (Cook et al., 1974; McLennan and Darby, 1980).

The state of the herpes genome in latently infected cells

In the 1960's Roizman introduced two alternate hypotheses to explain the persistence of HSV in nervous tissue (Roizman, 1965). The dynamic state hypothesis predicted a small number of cells would constantly replicate virus, but that infection would be localized by humoral or cell mediated immune mechanisms or through the action of interferon. Alternatively, the static state hypothesis involved conservation of the viral genome in a non-replicating state, either as an extrachromosomal element or integrated into the cellular DNA of the host.

Several lines of evidence argue against the dynamic state hypothesis. Latent infection has been established using temperature-sensitive mutants of HSV in animals hosts whose body temperature is restrictive for virus replication (Lofgren et al., 1977; McLennan and Darby, 1980; Watson et al., 1980). In addition, it has been shown that antiviral drugs which inhibit virus replication are

incapable of eliminating latent virus (Blyth et al., 1980; Field et al.,
1979; Klein et al., 1979). Lastly, kinetic hybridization studies
performed during the acute and chronic stages of HSV infection have
failed to demonstrate the presence of HSV mRNA at the latent stage. In
constrast, mRNA could be dectected during the acute stage of infection
and HSV DNA could be measured at the level of 1.2 - 2.0 genome
equivalents per cell at the acute stage and 0.11 ± 0.03 genome
equivalents per cell at the latent stage.

The only evidence which supported the dynamic state hypothesis was
the detection by ultrastructural methods, of rare neurons within the
trigeminal ganglia of latently infected rabbits which contained
morphologically complete virions (Baringer and Swoveland, 1974).
However this finding did not discount the possibility that spontaneous
reactivation had occurred within tissues being examined.

The above observations support the static state alternative, though
recent findings have challenged the notion that the viral genome is
completely quiescent while in the latent state. Galloway et al. (1979,
1982) have detected virus-specific transcripts within human sensory
ganglia. In studies by Green et al. (1981), indirect immuno-
fluorescence assays have revealed the presence of an immediate-early
polypeptide V_{mw} 175 (ICP 4) within cells of the trigeminal ganglia of
mice at the latent stage of infection. However, the transcripts
detected by Galloway et al. (1982) do not map in the region encoding ICP
4. The results from these two laboratories, while not in direct
agreement, do suggest that limited viral expression may be requisite to
or a consequence of maintenance of latent genomes.

Several studies have attempted to elucidate the precise molecular

relationship between host and viral DNAs. These studies have been hampered by the low proportion of latently infected cells per ganglion (estimated as 0.1% by Walz et al., 1974) and consequently by the small amounts of HSV nucleic acids present in the tissue of latently infected hosts (Puga et al., 1978). In addition, hybridization methodologies will most likely be affected by the homology of HSV DNA sequences originating from the reiterated regions of the genome to mammalian cell DNA (Peden et al., 1982; Puga et al., 1982).

Nevertheless, Rock and Fraser (1983) have recently been successful in defining the state of the herpes genome within the brain of latently infected mice by Southern blot hybridization analysis. When total virus DNA was used as a hybridization probe, complete genomes were shown to be present. However, when cloned probes representing the internal joint region were used, both internal and terminal repeats were detected at the acute stage of infection, but only DNA from the internal repetitive regions was detected at the latent stage. These results suggested the presence of "endless" DNA within latently infected cells, either in the form of closed-circular plasmids, long circular concatamers or linear molecules randomly integrated into host DNA. This was the first indication that in the latent stage viral DNA may exist in a different molecular configuration from that found at the acute stage. These findings must be interpreted with caution since the source of latent genomes, the brainstem, is a structure from which virus is not frequently reactivated. It will be of interest to see if the conclusions of Rock and Fraser (1983) apply to HSV DNA within sensory ganglia where the presence of latent virus has been proven by in vitro reactivation.

Immune control of herpesvirus latency

Though the precise mechanisms responsible for persistence of HSV in the host are unknown, several studies have dealt with the role of the immune response in establishment, maintenance and reactivation of latent virus.

Openshaw et al. (1979) have shown that in T-cell deficient (nude) mice inoculated with HSV, the ganglionic viral titer reaches high levels and all animals succumb to acute infection. Additional studies using nude mice demonstrated that anti-HSV antibody administered in high titer failed to eliminate acute ganglionic infection (Rager-Zisman and Allison, 1976). These studies implicated a cell-mediated immune response or possibly T-cell induction of humoral immunity in the elimination of productive ganglionic infection and subsequent establishment of the latent state. Studies by Sokawa et al. (1980) have indicated the transition from the acute to latent stage within animal ganglia is influenced by interferon; interferon-induced suppression of HSV replication at the acute stage occurred before the onset of antibody synthesis.

There have been several conflicting reports concerning the role of immune mechanisms in resistance to initial infection with HSV or to reinfection with either homologous or heterologous herpes simplex viruses. Walz et al., (1976) have shown immunization of mice to HSV-1 provides some protection in reducing both the severity of initial infection and the proportion of ganglion cells which become latently infected. Price et al. (1975) reported that substantial protection was achieved when animals were immunized and challenged with HSV-1 but no protection was afforded by immunization with HSV-1 and challenge with

HSV-2. In contrast, McKendall (1977) found that immunization with HSV-1 confered resistance to both acute and latent infections with HSV-2.

Studies by Klein et al. (1978) have demonstrated that HSV-infected nude mice with evidence of latent infection in spinal ganglia failed to develop latent HSV infections in trigeminal ganglia upon re-infection in the orofacial region. However, HSV-infected and phosphonoacetic acid (PAA) treated mice without evidence of latent infection in spinal ganglia were resistant to reinfection in the lumbar region, but not to reinfection in the orofacial area. It was concluded that extensive lesions in the epithelial surface at reinoculation may result in local accumulation of antibody and immunocompetent cells which prevent virus from entering nerve endings and establishing latent infections.

More recently, Centifanto-Fitzgerald et al. (1982) have reported that primary infection of rabbits and establishment of ganglionic latency with one strain of HSV-1 precluded colonization of ganglia with different challenging strains of HSV-1 applied at the same peripheral site. However, superinfecting viruses were shown to produce significant local infections. These workers utilized the highly-sensitive method of restriction enzyme analysis to show that only the initial infecting virus strain colonized the ganglia; no trace of superinfecting strains could be found in viruses recovered from the ganglia of doubly infected animals. It was concluded that the mechanism of protection of the ganglia is likely to be immunological, though its precise nature remains unclear.

In contrast to results reported previously Centifanto-Fitzgerald (personal communication, 1983) reported superinfecting, genetically dissimilar strains of HSV-1 could colonize the ganglia of rabbits if the

dose of the initial infecting strain was below 10^5 PFU. Thus it is possible that characteristics of initial infection may effect the pathogenesis of exogenous reinfections.

Investigations concerning the maintenance of HSV latency have implicated the involvement of antiviral IgG. In 1974, Stevens and Cook showed that viral DNA synthesis was inhibited in latently infected neurons within ganglia which were transplanted in Millipore chambers to uninfected mice passively immunized with specific immune IgG, but not in mice given non-immune IgG. Recently, Cook and Stevens (1983) have demonstrated IgG influences the pathogenesis of HSV in mice resistant to HSV infection. Following footpad inoculation, restriction of virus replication was shown to occur at the level of spinal ganglia where infiltration of IgG-bearing mononuclear cells was observed. This phenomenon was more marked in resistant than in susceptible animals. Nevertheless, other studies have demonstrated specific HSV antibody or interferon fail to prevent in vitro reactivation of virus from latently infected ganglia (Openshaw et al., 1979; Wohlenberg et al., 1979).

In vivo studies have shown that in animals passively immunized with rabbit anti-HSV serum following peripheral virus inoculation, latent virus is maintained despite declining and ultimately undetectable levels of neutralizing antibody. (Openshaw et al., 1981). It was concluded that persistence of virus in the absence of neutralizing antibody is due to immunological tolerance or alternatively, to insufficient antigenic stimulation needed to effect antibody production.

Reactivation of latent virus has been achieved in vivo in the presence of antibody following irradiation or cyclophosphamide treatment of latently infected immunocompetent mice (Openshaw et al., 1979).

Similar results have been obtained by treating passively immunized mice with 6-hydroxydopamine following intraocular injection with HSV (Price, 1979). The above findings suggest immunosuppression has a direct effect on the induction of latent virus. Kastrukoff and co-workers (1981) have shown that massive immunosuppression resulting in the removal of both radiation-sensitive and radiation-resistant memory T cells results in expression of infectious virus within brain tissue of mice carrying latent HSV within their trigeminal ganglia. They concluded that conditions of physiologic stress may cause a transient, but specific defect in HSV-specific cell-mediated immunity which could result in the emergence of infectious virus in the CNS despite the persistence of specific circulating antibody.

However, experimental data has failed to resolve the paradox that most human subjects with a clinical history of recurrent HSV infections have both high antibody titers to the virus (Douglas and Couch, 1970; Rand et al., 1977) and elicit lymphoycte responses to HSV antigens (Corey et al., 1978; Rosenberg and Notkins, 1974; Russell, 1974; Russell et al., 1975). The possiblity has been raised that the type, severity and pattern of recurrent herpetic disease in animals and man may be reflective of genetically determined virulence and pathogenic characteristics of specific virus strains as well as of host immune factors (Centifanto-Fitzgerald et al., 1982).

Aims of the project

The following studies address problems concerned with the interaction of latent HSV with human cells of neurological origin. An in vitro system of virus reactivation was utilized to detect viral genomes in human tissue and to isolate spontaneously expressed infectious virus from several neuroanatomical sites. Specificially, these questions were posed:

1. What factors affect the rate of in vitro reactivation of latent HSV from the trigeminal ganglia obtained from unselected human cadavers?

2. Can latent HSV be recovered from sites other than sensory and autonomic ganglia, such as the trigeminal nerve root?

3. Can non-reactivable HSV or HSV-specific genetic information latent in human neurological tissue be detected by the use of genetic probes in the form of ts mutants of HSV-2?

4. Is spontaneous expression of HSV from explant cultures of human trigeminal ganglia prevented by the continuous presence of the antiviral agent acyclovir?

5. Can multiple strains of latent HSV-1, as defined by variability in the number and location of specific restriction enzyme cleavage sites, be recovered from individual human hosts?

CHAPTER II

MATERIALS AND METHODS

Cells and viruses

Baby hamster kidney clone 13 (BHK C113) cells were obtained from the American Type Culture Collection and grown as monolayers in Eagle's minimal essential medium (EMEM) supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% fetal bovine serum (FBS). Cells were incubated in 5% CO_2, 95% air at 37° C.

Laboratory prototype virus strains HSV-1 Glasgow strain 17 (Brown at al., 1973) and HSV-2 strain HG52 (Timbury, 1971) and HSV-2 ts mutants ts 1, ts 2, ts 5 and ts 9 (Timbury, 1971) were supplied by J.H. Subak-Sharpe, Institute of Virology, Glasgow. ts 1, ts 2, ts 5 and ts 9 were derived from HSV-2 HG52; the permissive (PT) and non-permissive temperatures for the ts mutants are 31^oC and 38.5^oC, respectively. The physical map locations of ts 1, ts 5 and ts 9 have been determined by intratypic marker rescue and analysis of HSV-1/HSV-2 intertypic recombinants (Chartrand et al., 1981) and correlate almost exactly with those assigned by genetic analysis (Timbury and Calder, 1976). Ts 1, ts 2, ts 5 and ts 9 all map within the U_L segment of HSV-2 and are not clustered (Chartrand et al., 1981; Timbury and Calder, 1976). They are in different complementation groups though the precise nature of their genetic lesions is unknown (Timbury, 1971). Additional biochemical and genetic properties have been reported elsewhere (Timbury, 1971; Timbury and Calder, 1976).

Removal of human trigeminal ganglia and trigeminal nerve roots

The left and right trigeminal ganglia were obtained from unselected human cadavers at the time of postmortem examination (under

25 hours from time of death) by the method of Warren et al. (1977).
After removal of the brain, the dura mater in the area of the
trigeminal nerve root was wiped with sterile gauze soaked in 70%
alcohol. The dura matter was then dissected off the superior surface
of the ganglia. The ganglia appeared as yellow crescents in sharp
contrast to the white nerve root and peripheral nerves. A
crescent-shaped incision was made proximally and distally to the
ganglia. The ganglia were then separated from the cavum trigeminale by
dissecting them free from the underlying connective tissue. To remove
left and right trigeminal nerve roots, a cresent-shaped incision was
made just proximal to the ganglia and the nerve roots were dissected
free from connective tissue. Each ganglion and nerve root was then
immediately immersed into a sterile vial containing EMEM. The wet
weight of each trigeminal ganglion was approximately 250 mg.

Growth of human neurological tissue.

Each ganglion or nerve root was finely minced with sterile
scalpels and the tissue suspension divided equally between 25 cm^2
plastic tissue culture flasks. Ganglion tissue was divided into five
or ten 25 cm^2 flasks providing a total surface growth area of 125 cm^2
or 250 cm^2, respectively. Each trigeminal nerve root was divided into
five 25 cm^2 flasks. Tissue fragments were overlayed with EMEM
supplemented with L-glutamine (2 mM), penicillin (100 units/ml),
streptomycin (100 µg/ml) and 50% fetal bovine serum (FBS). All flasks
were incubated in 5% CO_2, 95% air at $37^{\circ}C$. After 5-7 days, the flasks
were observed for cell recovery and growth. The culture medium was
then replaced with EMEM supplemented with 20% FBS and the remaining
additives.

Rate of recovery of latent HSV from human trigeminal ganglia

HSV was recovered from 33 of 82 (40.2%) of individuals. Maximal recovery rate of HSV occurred when cultures were initiated within 12 hours of death on 250 cm^2. These conditions resulted in recovery of virus from 14 of 22 (63.6%) cases. When the cultures were initiated within 12 hours of death of the patient but onto a total area of 125 cm^2 rather than 250 cm^2, the recovery rate dropped to 11 of 23 (47.8%) cases. If the monolayers were initiated onto 250 cm^2, but not until 13-25 hours after death, the recovery rate was approximately the same at 8 of 19 (42.1%) cases. The lowest recovery rate, 0 of 18 (0%) cases, occurred when cultures were established 13-25 hours after death onto 125 cm^2.

Thus, if ganglia were explanted within 12 hours of death difference in surface area available for cell growth did not affect the HSV recovery rate to a statistically significant degree; however if explant cultures were established over 12 hours after death, the rate of HSV recovery increased significantly (χ^2 = 7.34, 1 df p<0.01) when the 250 cm^2 area was utilized.

Twenty-five of 45 (55.6%) cases established from 4-12 hours from death yielded spontaneous HSV, whereas 8 of 37 (21.6%) cases established from 13-25 hours from death were positive for spontaneous expression of virus. The increased rate of virus recovery in the group of ganglia obtained under 12 hours, relative to those obtained over 12 hours was statistically significant (χ^2 = 8.36, 1 df, p<0.01).

Growth of trigeminal ganglion cultures in the presence of acyclovir

The left and right trigeminal ganglia derived from 23 individuals were fragmented as described above and cultured in EMEM plus 50% FBS

with 10 μM acyclovir (ACV; Burroughs Wellcome Co., Research Triangle Park, North Carolina). After 5-7 days culture medium was replaced with the above medium containing 20% FBS. Cultured ganglia were maintained for 30-45 days as described above. Eighteen of 23 cultures released from ACV were rinsed twice in medium without ACV before refeeding with medium without drug. Cultures were monitored for the appearance of virus cytopathic effect and processed as mentioned below.

Virus isolation

Explantation cultures were monitored three times per week up to 45 days for the appearance of viral cytopatric effect (CPE) typical of HSV. After CPE progressed to involve 90% of the cellular monolayer virus was harvested by centrifugation of cells and medium at 2000 rpm for 10 min. at $4^{o}C$. Pellets were resuspended in 1.0 ml of supernatant fluid and sonicated for 30 sec. Primary virus isolates were stored at $-70^{o}C$ prior to passaging.

0.5 ml of the primary virus isolate suspension was passaged onto confluent monolayers of baby hamster kidney clone 13 (BHK Cl13) cells in 150 cm^2 tissue culture flasks. Virus was allowed to adsorb to the cells for 60 min at $37^{o}C$. Flasks were refed with supplemented EMEM plus 5% FBS. Cells were allowed to incubate for 48-72 hours or until CPE progressed to 90%. Flasks were subjected to one cycle of freeze-thawing. The contents of the flask were harvested by centrifugation at 2000 rpm for 10 min. at $4^{o}C$. pellets were resuspended into 2.0 mls of supernatant fluid and aliquots of 0.5 ml were placed in sterile vials prior to freezing at $-70^{o}C$.

Virus nomenclature

Single passage ganglion or root isolates derived from individual

cadavers were designated by the same arabic numbers. Isolates recovered from separate cultures derived from the same individual were given alphabetical letters in order of their recovery. Thus each isolate had both a number and a letter. Where isolates were plaque-purified an additional number following the letter indicated an independently derived subclone. Thus isolates 31A and 31B were derived from the same individual, but from different cultures; isolates 31A1 and 31A2 were subclones derived from the same culture.

Wild-type isolates rescued after superinfection with HSV-2 ts mutants (described below) were designated by the autopsy case number from which the culture was derived followed by the number of individual culture which was superinfected. An alphabetical letter indicated a plaque-purified clone. For example, isolate 444-3B was rescued following superinfection of culture 3 from autopsy case A444/80 and represented one of several plaque-purified subclones.

Virus plaque purification

Virus suspensions were plated on 50 mm petri dishes of confluent BHK C113 cells so as to produce approximately 10 plaques per dish. Virus was adsorbed for 1 hour at $37^{\circ}C$, or at $31^{\circ}C$ or $38.5^{\circ}C$ if temperature- sensitivity of strains was to be determined. Plates were overlayed with EMEM with additives plus 2% FBS and incubated for 2-3 days or until plaques were visible at the appropriate temperature. Prior to picking plaques, cell sheets were washed twice with sterile phosphate-buffered saline (PBS). One or two well-separated plaques per dish were picked into 0.5-1.0 ml EMEM + 2% FBS and sonicated for 30 sec. 0.1 ml of this suspension was used to infect monolayers of BHK C113 cells at $37^{\circ}C$, $31^{\circ}C$ or $38.5^{\circ}C$. This procedure was repeated 2-3

times.

Virus titration

Virus suspensions were titrated by a modification of the method described by Russell (1962). 0.1 ml of serially diluted virus suspension was used to infect confluent monolayers of BHK C113 cells in 50 mm petri dishes. Adsorption was for 1 hour at $37^{o}C$, or where temperature-sensitivity of isolates was to be determined, at $31^{o}C$ and $38.5^{o}C$ to quantitate total virus yields, and yields of ts+ virus, respectively. After 1 hour, plates were overlayed with EMEM + 2% pooled human serum to restrict plaque spreading. Plates were incubated at $37^{o}C$, $31^{o}C$ or $38.5^{o}C$ for 2-3 days. Monolayers were fixed in formal saline, stained with Giemsa and plaques were counted.

Superinfection of ganglion and control cultures with HSV-2 temperature-sensitive mutants

Ganglion cultures which failed to release HSV spontaneously were infected with individual temperature-sensitive (ts) mutants of HSV at an input multiplicity of one plaque-forming unit (PFU) per cell. Parallel cultures were infected with one PFU per cell of HSV-2 HG52 or mock infected. Control BHK C113 and primary fetal brain cultures were similarly infected. For infection, virus was was adsorbed for 1 hour at $37^{o}C$ for cells subsequently incubated at $31^{o}C$ and at $38.5^{o}C$ for cells subsequently incubated at $38.5^{o}C$. After 1 hour unadsorbed virus was removed by washing monolayers with sterile PBS. Flasks were refed with EMEM plus 20% FBS.

Cells were incubated for 24 hours at $38.5^{o}C$ to allow complement-ation to occur or at $31^{o}C$ to allow for recombination between superinfecting virus and putative resident genomes. Between 6 and 38

ganglion cultures obtained from a single cadaver were infected with at least 3, and in most cases 4, individual ts mutants. Progeny virus was assayed at 31°C to determine total yield and at 38.5°C to detect any ts+ virus in the yield. ts+ viruses recovered from ganglion cultures following superinfection with HSV-2 ts mutants were passaged on BHK Cl13 cells and plaque purified twice before subsequent restriction enzyme analysis. ts+ strains and viruses spontaneously released from ganglia cultures were digested with restriction endonucleases, Hind III, Kpn I and Bam HI according to the following method.

Virus identification by restriction endonuclease analysis

Viral DNA was prepared by the method of Lonsdale (1979). BHK Cl13 cells seeded in 1.6 cm Linbro wells containing EMEM and the 10^{-6} M phosphate supplemented with 1% FBS (PIC) were incubated for 24 hours at 37°C. The medium was removed and each well was infected with virus at an input multiplicity of 10 plaque forming units (PFU) per cell and allowed to adsorb for 1 hour at 37°C. Cells were washed with PIC, and 0.5 ml of PIC was added to each well. Linbro plates were incubated at 37°C for 2-4 hours before 50 µCi of [^{32}P] orthophosphate (1 mCi = 37 MBq) was added to each well. Incubation was continued overnight at 37°C. After the appearance of viral CPE (usually within 24 hours), the contents of each well were harvested. A 0.5 ml volume of 5.0% (w/v) sodium dodecyl sulfate was added to each well and mixed by swirling. The mixture in each well was transferred to glass centrifuge tubes and mixed, by inversion, with 1 ml of phenol saturated with 75 mM NaCl and 50 mM EDTA, pH 8.0. Tubes were placed in an ice water bath for 10 min and centrifuged for 10 min at 2000 rpm at 4°C. The aqueous phase was removed and transferred to a glass centrifuge tube. The nucleic acids

were precipitated by the addition of two volumes of ethanol and immediately pelleted by centrifugation for 10 min at 2000 rpm at 4°C. The supernatant was discarded and the nucleic acids were resuspended by gentle shaking for 1-2 hours at 37°C in 0.2 ml of distilled water containing 20 µg of RNase A and 200 units of RNase T_1. A 10 µl volume of the redissolved nucleic acids was transferred to a Whatman's No. 1 filter papér disc and air-dried. Paper discs were washed three times in 5% trichloroacetic acid - 0.1 M sodium tetraphosphate, three times in absolute ethanol, and once in acetone and counted in toluene - 5% PPO.

Samples containing equal numbers of RNase-resistant counts in 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 100 µg/ml bovine serum albumin in a final volume of 30-50 l were incubated individually with 5-10 units of restriction enzymes Hind III, Kpn I and Bam HI (Bethesda Research Laboratories) for 4 hours at 37°C. The reactions were stopped by the addition of 10 µl of 60% sucrose in 0.2 M EDTA, pH 7.5, containing bromophenol blue.

Agarose and polyacrylamide gel electrophoresis

Digests were analyzed after overnight electrophoresis on 0.6% - 1.2% agarose gels at 2 V/cm. The gel and tank buffer consisted of 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA. Gels were dried on glass plates or vacuum dried with a Bio-Rad gel dryer and exposed with intensifying screens to preflashed Kodak X-omat RP-1 film at -70°C for varying lengths of time.

For resolution of low molecular weight Bam HI and Kpn I digestion fragments, digests were electrophoresed in 3.5% polyacrylamide gels by the method of Laemmli (1970). Gels were formed from a solution

containing 30% w/v of 29:1 w/w acrylamide: N, N^1 -methylene

bisacrylamide and run at room temperature at 2V/cm overnight. The gel

and tank buffer was 36 mM Tris, 30 mM NaH_2PO_4 and 1 mM EDTA. Gels were

vacuum dried and autoradiographed at -70°C using pre-flashed Kodak

X-omat RP-1 film with an intensifying screen. Exposure was for 4-7

days.

Southern transfer and hybridization

DNA fragments electrophoresed through agarose gels were

transferred to nitrocellulose filters (Schleicher and Schuell Co.)

essentially by as described by Southern (1975). To denature DNA, the

agarose gels were washed twice in 0.25M HCl for 5-10 min, and twice in

0.5M NaOH for 10-15 min. The gel was neutralized by two 30 min

treatments in 0.5M Tris (pH 7.4), 3M NaCl prior to transfer in 20 x SSC

(1 x SSC is 0.15M NaCl, 0.015M sodium citrate). For blotting onto

nitrocellulose filters, gels were placed on top of layers of Whatman

3MM filter paper. Nitrocellulose wetted in 2 X SSC was placed on top

of the gel. 3MM paper towels were placed on top of the nitrocellulose

filter onto which a weight was placed. Transfer proceeded overnight.

The filter was dried between two sheets of 3MM paper and baked at 80°C

for 2 hours prior to hybridizations.

Nitrocellulose filters were placed in heat-sealable plastic bags

prehybridized in a 42°C waterbath for 4-6 hours in 15 ml pre-

hybridization buffer. The prehybridization buffer consisted of 40 ml

deionized formamide, 20 ml 20 x SSC, 8 ml 50X Denhardt's solution (1%

bovine serum albumin, 1% polyvinyl pyrrolidine, 1% Ficol MW 400K) 4 ml

1M $NaPO_4$ (pH 6.5) and 8 mg salmon sperm DNA. The filters were

hybridized with $1.6-3.0 \times 10^7$ cpm (0.75-1.5 µg) of ^{32}P - labeled

denatured nick translated probe (see below) in hybridization buffer (4 parts prehybridization buffer: 1 part 50% dextran sulfate) at 42°C overnight.

The filters were washed with gentle shaking 4 times for 5 min in 2 x SSC, 0.1% SDS at room temperature, and 3 times for 15 min in 0.1 x SSC, 0.1% SDS at 50°C. Filters were dried at 50°C for 1 1/2 hours prior to autoradiography.

Preparation of radiolabeled hybridization probes

Radioactively labeled probes were prepared using a nick translation kit (Amersham). 1.5 µg of purified plasmid DNA containing HSV-1 KOS fragments Bgl II-jl and Bgl II-k (obtained from W.C. Leung, University of Alberta) were individually incubated in 20 µl solution 1 [100 µM each dATP, dTTP and dGTP in buffer containing Tris/HCl (250 mM) pH 7.8, MgCl$_2$ (25 mM) and β - mercaptoethanol (50 mM)], 60 µCi α ^{32}P-dCTP (specific activity 3000 Ci/m mole, Amersham) and 10 µl solution 2 (5 units DNA polymerase 1 and 100 pg DNase 1 in a buffer solution containing Tris/HCl, pH 7.5, magnesium chloride, glycerol, bovine serum albumin and sterile deionized water) in a total volume of 100 µl. The reaction mixture was incubated at 15°C for 2 hours. The reaction was terminated and unincorporated nucleotides removed by immediate addition of 1/10 volume 3M NaAc, 20 µg tRNA and 2.5 volumes ethanol followed by precipitation in a dry ice/ethanol mixture for 5 min. The mixture was centrifuged in an Eppendorf Microfuge at 12,000 x g for 5 min and the pellet was resuspended in 100 µl sterile water. The solution was boiled for 5 min, chilled on ice and added quickly to hybridization buffer. The nick translated DNA probes had specific activities of 1.6-6.0 x 10^7 cpm/µg.

CHAPTER III

RECOVERY OF LATENT HERPES SIMPLEX VIRUS FROM HUMAN TRIGEMINAL NERVE

ROOTS

INTRODUCTION

Latent HSV has previously been recovered from several

neuroanatomical sites of human cadavers. These include the trigeminal

(Baringer and Swoveland, 1973; Bastian et al., 1972; Plummer, 1973;

Warren et al., 1977) superior cervical, vagus (Warren et al., 1978) and

sacral ganglia (Baringer, 1974).

In animal model systems, migration of HSV from peripheral sites to

the central nervous system involves the progression of virus infection

from mucocutaneous surfaces, to the trigeminal nerve, ganglion, root and

trigeminal tract within the brainstem (Baringer and Griffith, 1970;

Goodpasture and Teague, 1923; Kristensson et al., 1978; Townsend and

Baringer, 1978). The present study was undertaken to determine whether

latent HSV could be isolated from additional human neuroanatomical

structures especially those proximal to known sites of latency. The

trigeminal nerve root was of particular interest as latent virus located

within this site could be a source of infectious or possibly defective

HSV genomes involved in the pathogenesis of central nervous system

disease.

Results

Latent herpes simplex virus was isolated from explant cultures of

trigeminal nerve roots derived from 7 of 47 human cadavers (Table 4).

Virus specific cytopathic effect (CPE) appeared in the monolayers 6 to

22 days following establishment of nerve root cultures. In one case

(A060/81) virus was isolated from both the left and right trigeminal

nerve roots, whereas in the remaining 6 cases virus was recovered from either the left or right nerve root.

In 2 of 7 individuals where latent virus was recovered from trigeminal nerve roots, HSV was isolated from explant cultures of the corresponding trigeminal ganglia (cases A060/81 and A079/82). In one of these cases (A079/82), latent HSV was recovered from a ganglion culture following removal of the antiviral drug, acyclovir (ACV) (see Chapter VI).

Ganglion cultures derived from the remaining 5 individuals were negative for spontaneous expression of latent HSV; however, in 2 cases ganglia were cultured in the continuous presence of acyclovir for 30 days. In one of these 2 cases, ganglia were released from ACV and cultured in the absence of drug, but latent HSV was not recovered. Trigeminal ganglia derived from the other 3 individuals failed to spontaneously express HSV. (Table 4).

Virus isolates recovered from explant cultures of human trigeminal nerve roots, and where available corresponding trigeminal ganglia were, identified as HSV type 1 (HSV-1) by restriction enzyme analysis of viral DNA (Fig. 1). Digestion of viral DNA with Bam HI revealed all isolates had an overall restriction enzyme profile characteristic of prototype HSV-1 Glasgow strain 17. None of the enzyme patterns resembled that of prototype HSV-2 HG52.

Virus isolates derived from different individuals had distinguishable Bam HI profiles with the exception of isolates 31A and 31B. Isolates 31A and 31B derived from the left and right trigeminal nerve roots, respectively, of the same individual exhibited different restriction enzyme patterns. Ganglion isolates derived from this

individual also displayed variability in the number and location of restriction enzyme cleavage sites (see Chapter VII, Fig. 13-15). Isolates 42A and 42B recovered from the right trigeminal nerve root and right trigeminal ganglion, respectively had identical Bam HI restriction enzyme profiles.

Discussion

The trigeminal nerve complex is divided into peripheral nerves, ganglia and nerve roots. Peripheral nerves join the ganglia to mucocutaneous surfaces, whereas trigeminal nerve roots join the ganglia to the central nervous system brainstem. Histologically, trigeminal ganglia differ from trigeminal nerve roots in that the ganglia contain the majority of sensory neuron cell bodies, whereas the nerve roots are composed mainly of Schwann cells with an occasional ectopic sensory neuron located at the distal end of the nerve root.

The isolation of HSV-1 from trigeminal nerve root cultures derived from 7 of 47 unselected human cadavers indicated that clinically latent HSV is not confined to autonomic and sensory ganglia. However, it appeared as if only a small percentage of individuals harbors latent virus within the trigeminal nerve root.

Because of experimental design it was difficult to determine if all individuals positive for latent virus within nerve roots also harbored latent virus within the trigeminal ganglia. Two of 7 individuals had latent HSV in both sites; however, where culture conditions allowed for spontaneous expression of HSV from corresponding ganglia, 4 of 7 individuals remained negative for latent virus within this site. Cultures derived from the trigeminal ganglia of the remaining individual failed to express virus, however the continuous presence of acyclovir in

these cultures most likely prevented in vitro reactivation of latent HSV. The above results suggested that within the same human host latent HSV may reside in trigeminal nerve roots irrespective of the presence of virus in trigeminal ganglia. However, further experiments where trigeminal nerve roots are cultured along with corresponding trigeminal ganglia under identical conditions must be performed to substantiate this suggestion.

It is unlikely "stress" of death activated latent HSV from sensory neurons causing spread of infectious virus into the nerve root and re-establishment of a latent state in nerve root cells. Such a phenomenon has not been observed in experimental animals and probably does not occur in humans between the time of death and removal of ganglia at autopsy.

The bulk of evidence, though indirect, suggests that latent HSV is associated with neurons within sensory ganglia (Green et al., 1981; McLennan and Darby, 1980; Stevens, 1975). In the ganglia of both latently infected animals and humans, it has been estimated that only a very small fraction of ganglion cells contains HSV nucleic acids or reactivable virus (Galloway et al., 1979, 1982; Puga et al., 1978; Walz et al., 1976). In addition, only a few cells within human trigeminal ganglion explant cultures have ultrastructural and electrophysiological properties characteristic of neurons (Kim et al., 1979). In studies described here, the possibility that occasional ectopic neurons in the trigeminal nerve root of humans were the source of latent HSV cannot be discounted. However, the likelihood that a neuron was ectopic, and also both latently infected and viable in culture is exceedingly small.

Recovery of HSV from trigeminal nerve roots suggests that latent

virus may be present in cells types other than ganglionic neurons. It is possible HSV resides in myelin-producing Schwann cells of the peripheral nervous system (PNS), in axons passing from the ganglionic sensory neurons to the central nervous system (CNS) or in sparse astrocytes or oligodendroglia bordering the PNS/CNS junction. The identification of cell types present in trigeminal nerve root explant cultures by specific staining procedures or use of monoclonal antibodies was not performed. However, at present, most available identification techniques for human neural cells require the fixation or processing of cells. Such treatment would subsequently render cells non-viable in culture or arrest the replication of host or viral DNA. This precludes definitive association of cell types present within a given culture with in vitro reactivation of HSV.

In experimental animal systems, centripetal migration of HSV from sensory ganglia to central nervous system tissue following peripheral inoculation of HSV has been demonstrated by the appearance of distinctive lesions in the trigeminal root entry zone (Kristensson et al., 1978, 1979; Townsend and Baringer, 1978). Biochemical and electron microscopic investigations have indicated ascending infection of the trigeminal tract occurs by retrograde intra-axonal transport of virus (Baringer and Swoveland, 1974; Cook and Stevens, 1973; Kristensson, 1975; Kristensson et al., 1971, 1974). Therefore, infectious virus present in axons within the trigeminal nerve root could be a source of virus which becomes latent in cells within this site.

It remains to be determined if latent HSV harbored in sensory ganglia or nerve roots of humans, in addition to migrating centrifugally to mucocutaneous surfaces to cause recurrent lesions, may also travel

centripetally to the central nervous system to evince neurological
disease.

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TABLE 1   RECOVERY OF LATENT HERPES SIMPLEX VIRUS FROM THE TRIGEMINAL NERVE ROOTS OF HUMAN CADAVERS

Case number	Age and sex	Cause of death	Recovery of HSV from trigeminal nerve roots	Root expressing virus	Days culture to spontaneous virus expression	Recovery of virus from coresponding trigeminal ganglia
A036/81	66M	lung cancer	-	-	-	-[a]
A035/81	55M	leukemia	-	-	-	-
A031/81	63M	carcinomatosis	+	right	12	-
A060/81	63F	pulmonary emboli	+	right, left	9, 12	+
A066/81	55M	lung cancer	-	-	-	-
A081/81	59M	cerebral infarction	-	-	-	-
A085/81	60F	breast cancer	+	left	6	-
A092/81	59M	prostate cancer	-	-	-	-[a]
A137/81	78F	myocardial infarction	+	left	11	-[a]
A130/81	35F	breast cancer	-	-	-	-
A132/81	68F	breast cancer	-	-	-	-
A142/81	60F	motor vehicle accident	-	-	-	-
A170/81	73M	cerebral vascular accident	-	-	-	-[a,b]
A172/81	82F	pneumonia	+	left	15	-
A186/81	72M	lung cancer	-	-	-	-
A187/81	17F	multiple sclerosis	-	-	-	-
A182/81	57M	shock	-	-	-	-[a,b]
A207/81	66M	metastatic carcinoma	-	-	-	-
A306/81	77M	mycosis fungoides	-	-	-	-[a]
A315/81	79M	renal failure	-	-	-	-
A316/81	44F	multiple sclerosis	-	-	-	-[a,b]
A321/81	57M	lung cancer	-	-	-	-
A325/81	90F	myocardial infarction	-	-	-	-
A337/81	52F	intracerebral hemorrhage	-	-	-	-
A338/81	75F	hydronephrosis	-	-	-	-[a,b]
A340/81	73F	carcinomatosis	-	-	-	-[a,b]

TABLE 1 continued

Case number	Age and sex	Cause of death	Recovery of HSV from trigeminal nerve roots	Root expressing virus	Days culture to spontaneous virus expression	Recovery of virus from trigeminal corresponding ganglia
A341/81	24F	cystic fibrosis	-	-	-	$-$a,b
A363/81	61M	myocardial infarction	-	-	-	-
A378/81	67M	heart failure	-	-	-	-
A423/81	21F	Down's syndrome	-	-	-	$-$a,b
A002/82	76F	heart failure	-	-	-	$-$a,b
A011/82	63F	heart valve replacement	-	-	-	-
A028/82	61M	cardiovascular arrest	-	-	-	$\pm$a,b
A079/82	78M	cardiopulmonary arrest	+	right	22	$-$a,b
A102/82	66M	sarcoma	-	-	-	a,b
A113/82	66M	myocardial infarction	-	-	-	$-$a
A120/82	60M	prostate cancer	-	-	-	-
A133/82	100M	pancreatic cancer	-	-	-	$-$a,b
A134/82	62M	lung cancer	-	-	-	-
A188/82	36F	cervical cancer	-	-	-	$-$a,b
A189/82	57M	lung cancer	-	-	-	$-$a,b
A191/82	36F	breast cancer	-	-	-	-
A311/82	76M	bladder cancer	+	left	8	-
A324/82	27F	cervical cancer	-	-	-	$-$a,b
A211/82	70F	heart failure	-	-	-	-
A401/82	65M	cardiac arrest	-	-	-	-
A409/82	31M	multiple sclerosis	-	-	-	-

Total cases expressing HSV in trigeminal nerve roots   7/47

a Ganglia were maintained in the presence of 10 μM acyclovir (ACV) for 30 days

b After release of 10 μM ACV, ganglion cultures were maintained in drug free medium for an additional 15-20 days
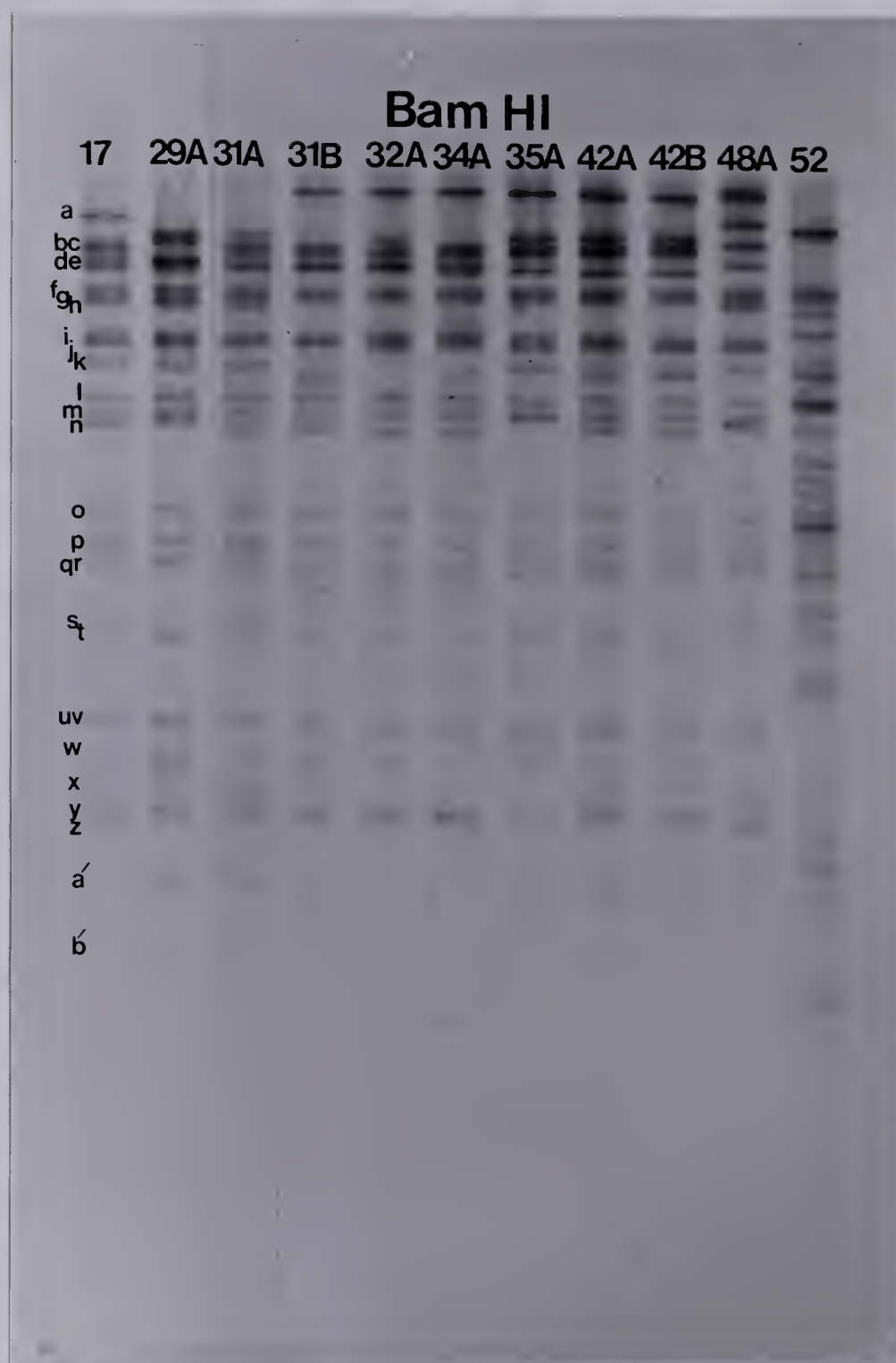
Figure 1

Autoradiograph of Bam HI digests of ^{32}P-labeled DNA of HSV isolated from human trigeminal nerve roots and corresponding trigeminal ganglia. HSV-1 17 and HSV-2 HG52 are laboratory prototypes. Strains 29A, 31A and 31B, 32A, 34A, 35A, 42A and 48A are root isolates from cases A031/81, A060/81, A085/81, A137/81, A172/81, A079/82, and A311/82, respectively. Strain 42B is a isolate derived from the right trigeminal ganglion from case A079/82.

CHAPTER IV

RECOVERY OF HERPES SIMPLEX VIRUS GENETIC INFORMATION FROM HUMAN

TRIGEMINAL GANGLION CELLS FOLLOWING SUPERINFECTION WITH HERPES SIMPLEX

VIRUS TYPE 2 TEMPERATURE-SENSITIVE MUTANTS

Introduction

In vitro culture techniques have enabled the recovery of clinically
latent HSV from the sensory and autonomic ganglia (Baringer, 1974;
Baringer and Swoveland, 1973; Bastian et al., 1972; Warren et al., 1977,
1978) of unselected human cadavers. Virus is usually recoverable from
40-60% of individuals (Baringer, 1975; Forghani, et al., 1977; Warren et
al., 1977), though immunological evidence indicates a much higher
prevalence of HSV infection (Forghani et al., 1977).

Spontaneous reactivation of HSV from human trigeminal ganglia
occurs from 7-45 days following explantation or co-cultivation with
indicator cells (Baringer and Swoveland, 1973; Warren et al., 1977).
Trigeminal ganglion cells which fail to release virus after 45 days in
culture have been shown to contain HSV genetic information capable of
complementing or recombining with superinfecting temperature-sensitive
(ts) mutants of HSV-1 (Brown et al., 1979). This observation suggested
some individuals may harbor virus in a non-reactivable form thus
accounting for the discrepancy between serological evidence of prior HSV
infection and the rate of HSV recovery from human cadavers.

In the present study, HSV-2 ts mutants have been employed as
genetic probes to detect the presence of putatative HSV-1 information in
ganglion cells which failed to express virus during the course of the
in vitro culture. Restriction enzyme analysis of ts+ virus rescued from

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ganglion cells following intertypic superinfection with HSV-2 ts mutants was performed to unambiguously distinguish between the following events: (1) activation or spontaneous expression of endogenous HSV genomes consequent to superinfection, (2) recombination between superinfecting ts mutants and resident HSV genetic information, or (3) reversion of superinfecting ts mutants to ts$^+$ virus. If activation or spontaneous expression of endogenous genomes occurred resulting viral progeny would have a restriction enzyme profile characteristic of HSV-1. Genetic recombination between superinfecting ts mutants and resident HSV genetic information would result in the generation of viral progeny with restriction enzyme profiles characteristic of intertypic (HSV-1 x HSV-2) recombinants. Finally, if reversion of superinfecting ts mutants to ts$^+$ virus occurred, viral progeny would have an HSV-2 restriction enzyme profile identical to that of the parental strain (HSV-2 HG52) from which the ts mutant originated.

Results

The origin of trigeminal ganglion cultures obtained from 36 randomly selected cadavers, along with information regarding spontaneous release of HSV and culture conditions prior to superinfection are listed in Table 5. Ganglia obtained from 17 individuals were maintained in culture for 28-62 days prior to superinfection (Table 5, Part A), whereas those from 19 individuals were cultured for 9-21 days before superinfection with HSV-2 ts mutants (Table 5, Part B). Cultures were divided into two groups to determine whether frequency of rescue of ts$^+$ virus by superinfecting mutants was different in cells superinfected early after explantation compared to those superinfected at later times.

Cultures from 3 of 17 individuals which were maintained in culture

28-62 days prior to superinfection and cultures from 12 of 19

individuals maintained in culture 9-21 expressed HSV spontaneously.

Thus cultures from 15 of 36 (42%) individuals spontaneously released HSV

which is within the 40-60% range observed by others (Baringer, 1975;

Warren et al., 1977). Spontaneous isolates were identified as HSV-1 by

restriction enzyme analysis (Chapter VII).

Trigeminal ganglion cultures which failed to express HSV

spontaneously were superinfected with single ts mutants of HSV-2 at an

input multiplicity of 1 PFU per cell. Cultures maintained 28-62 days

after explantation (Table 5, Part A) were infected at passages 1-3.

Twenty to 40 cultures from a single individual were infected with 3 or 4

individual ts mutants at both permissive ($31^{o}C$) or nonpermissive

temperatures ($38.5^{o}C$). Five cultures from 4 individuals yielded

positive results (Table 6A). Complementation was observed in a culture

from autopsy case A292/80. Complementation and rescue of ts[+] virus

occurred in cultures from cases A274/80, A276/80 and A292/80; rescue of

ts[+] virus occured in a culture from case A261/80. None of the ganglion

cultures derived from these 4 individuals expressed HSV prior to

superinfection.

Cultures maintained 9-21 days after explantation (Table 5, Part B)

were superinfected prior to passaging and, due to their restricted

numbers, were incubated only at the permissive temperature ($31^{o}C$). In

several instances, superinfection of negative cultures was performed

close to the time of in vitro reactivation of latent HSV from parallel

cultures, and in one case (A444/80) spontaneous HSV was expressed in a

mock-infected culture following superinfection of parallel cultures.

ts[+] virus was obtained from cultures from cases A386/80 and A444/80 in

this group (Table 6B).

ts$^+$ viruses rescued from cultures A386(9), A444(3), and A444(12) were subjected to two cycles of plaque purification at the nonpermissive temperature (38.5^{o}C). Plaque-purified isolates grew to approximately equal titers at permissive and nonpermissive temperatures. ts$^+$ virus was not recoverable from culture A261(2) upon subsequent passage. The reason for this is not known, and it can only be speculated that leak-through of ts 1 occurred in this ganglion culture though not in corresponding control BHK C113 or fetal brain cells.

Restriction enzyme analysis with Kpn I and Bam HI of spontaneous isolates from cases A386/80 and A444/80 along with ts$^+$ viruses rescued after superinfection of cultures with HSV-2 ts mutants is shown in Figs. 2 and 3.

Spontaneous isolates recovered from case A386/80 were uniformly HSV-1 and had Kpn I and Bam HI (Fig. 2) restriction enzyme patterns identical to one another and also to ts$^+$ virus recovered after superinfection with HSV-2 ts 1. None of the bands corresponded to HSV-2 fragments. Similarly isolates recovered from two cultures from case A444/80 following superinfection with ts 5 were identical by the criterion of number and location of restriction enzyme sites to endogenous HSV-1 spontaneously expressed (Fig. 3). In addition, isolates from cases A386/80 and A444/80 could be distinguished from HSV-1 17 and from one another. Intratypic variability was most clearly seen when comparing Bam HI restriction enzyme patterns.

Discussion

The present study has demonstrated the retrieval of HSV genetic information from latently infected human trigeminal ganglia by

superinfection of explant cultures with genetic probes in the form of HSV-2 ts mutants. Previously Brown et al., (1979) employed the technique of superinfection of latently infected human ganglion cells with HSV-1 ts mutants to demonstrate the presence of HSV specific genes within cells by complementation or recombination with input virus. In this study we have utilized HSV-2 ts mutants to more definitively characterize the interaction between superinfecting virus and resident HSV genomes by application of restriction enzyme technology.

Evidence of complementation or rescue of ts^+ virus was obtained from 8 cultures derived from 6 individuals; ganglia from 2 of these individuals contained latent HSV which reactivated in vitro prior to superinfection. Positive results were obtained with ts 1 and ts 5 though cultures from most cases were infected with ts 1, 2, 5 and 9. Since the functional nature of the ts mutations is not known, it is difficult to speculate on the selective interaction of ts 1 and ts 5 with latent genomes.

In the initial experiments of Brown et al., (1979) ganglia from 8 of 14 individuals which had been consistently negative for spontaneous release of virus were shown to contain uninducible genomes by super-infection with HSV-1 ts mutants. Negative ganglion cultures from an additional two individuals also yielded positive results, though parallel cultures had previously released HSV spontaneously. In experiments reported here, ganglion cultures from only 6 of 36 individuals which failed to release virus spontaneously were shown to harbor HSV information by genetic probing with HSV-2 ts mutants. Fewer positive results in this study suggest that efficiency of complementation and recombination between superinfecting virus and

resident genomes may be higher when homotypic rather than heterotypic viruses are used. Lower rescue frequencies could also be attributed to the longer culturing of ganglion cells prior to superinfection. Some cultures were maintained over 60 days and latently infected cells may have been diluted out and lost from cultures by passaging. In addition, many of the ganglia superinfected early after explantation spontaneously yielded HSV leaving fewer cultures to be genetically probed.

HSV-1 and HSV-2 share approximately 50% homology in their nucleotide sequences (Kieff et al., 1972) and both complementation and recombination between the two serotypes have been reported (Esparza et al., 1976; Timbury and Subak-Sharpe, 1973). Restriction enzyme analysis which unambiguously differentiates HSV-1 from HSV-2 (Hayward et al., 1975a; Skare et al., 1975) was applied to ts$^+$ viruses recovered from ganglia superinfected with HSV-2 mutants. Plaque-purified strains rescued after superinfection had restriction enzyme patterns identical to HSV spontaneously released from the ganglia of corresponding individuals. No HSV-1 x HSV-2 ts$^+$ recombinants or ts$^+$ revertants of superinfecting viruses were recovered. However, the possibility cannot be excluded that recombination between superinfecting ts mutants and resident genomes occurred resulting in the insertion of an HSV-2 DNA fragment responsible for a ts$^+$ phenotype but too small to cause alteration in adjacent HSV-1 Kpn I or Bam HI restriction enzyme sites.

Superinfecting ts mutants may have activated latent endogenous genomes, perhaps by providing a product essential for the stimulation of virus replication. Such a mechanism has been suggested in an in vitro model system of cells latently infected with HSV. Superinfection with heterologous (CMV) or partially homologous viruses (HSV-1 or HSV-2)

caused reactivation of parental HSV used to establish inapparent infection (Colbery-Poley et al., 1979; Wigdahl et al., 1981, 1982). In studies outlined here, identification of a viral product acting in trans and capable of inducing spontaneous viral expression could be facilitated by repeating the above experiments with infected cell supernatants or UV-inactivated virus.

Recently, a factor responsible for activating HSV α genes has been characterized (Batterson and Roizman, 1983). This α gene inducer was shown to be an HSV-1 or HSV-2 specific virion component located outside the capsid the suggested function of which is to enhance transcription of α genes. Whether the described factor is unique to isolated ts mutants or is universally present in virions of HSV-1 and HSV-2 is unknown. In experiments described here, it is conceivable that an identical or related inducer present in superinfecting virions activated expression of latent genomes.

Alternatively, reactivation of endogenous HSV-1 following superinfection with HSV-2 ts mutants may have been unrelated to the act of superinfection and may have been attributable to expression of latent genomes in temporal proximity to superinfection. This hypothesis is supported by the occurrence in one instance of spontaneous reactivation of HSV in a mock-infected culture. If superinfection were coincident to spontaneous expression, one would expect recombination to occur between endogenous wild-type genomes and superinfecting ts mutants. However, no selection system is presently available to distinguish between endogenous HSV-1 and putative HSV-1 x HSV-2 recombinants which may have been a minority component of the wild-type virus population.

Park et al., (1980) were successful in retrieving intertypic HSV

recombinants from HSV-transformed cells following superinfection with ts mutants of the alternative serotype. Restriction enzyme analysis demonstrated the isolation of recombinant viruses which spanned the genome as well as virus indistinguishable to that used in the original transformation. These positive results most probably were related to the content of HSV per cell. In contrast to HSV transformed cells, ganglia contain only a small number of latently infected cells undergoing limited expression (Galloway et al., 1979, 1982; Puga et al., 1978; Walz et al., 1976). Therefore, genetic interaction between latent genomes and superinfecting virus probably occurs very rarely.

Recently, Campione-Piccardo and Rawls (1981) reported the inability to rescue viral genes from human cells biochemically transformed by HSV-2 DNA by superinfection or transfection of cells with heterotypic virus or viral DNA. They suggested rescue experiments have limited applicability in the detection of small pieces of viral DNA.

The relatively low frequency of retrieval of virus information from human ganglion cells by superinfection techniques as reported here emphasizes the need to: (1) utilize more sensitive methods to detect viral products in latently infected cells, and (2) amplify in vitro the number of cells containing HSV information so that the molecular mechanisms responsible for latency can be better studied.

TABLE 2   ORIGIN OF TRIGEMINAL GANGLIA EXPLANT MONOLAYERS SUPERINFECTIONED WITH HSV-2 ts MUTANTS

Part A - Cultures maintained 28-62 days prior to superinfection

Autopsy case	Age and sex	Cause of death	Hrs to explantation	Spontaneous HSV release	Days to spontaneous virus expression	Days to superinfection	Passage at superinfection
A237/80	51F	carcinomatosis	11.5	+	16, 21	62	3
A239/80	48M	metastatic carcinoma	5	+	42	61	3
A248/80	68M	myocardial infarction	4.5	-	-	57	2
A253/80	16F	trauma	6	+	21, 27	50	3
A254/80	47M	liver cirrhosis	21.5	-	-	57	2
A255/80	54M	coronary artery disease	7.5	-	-	52	1
A261/80	56M	pneumonia	7	-	-	48	2
A266/80	74F	myocardial infarction	8	-	-	45	1
A271/80	84F	hepatoma	24	-	-	42	1
A274/80	51M	hepatic failure	3	-	-	38	1
A276/80	88F	bladder cancer	9	-	-	37	2
A286/80	62M	lymphoma	9	-	-	34	2
A288/80	63M	myocardial infarction	12	-	-	36	2
A291/80	66M	myocardial infarction	7	-	-	30	2
A292/80	68F	burn	8	-	-	30	2
A296/80	50F	carcinomatosis	24	-	-	28	1
A297/80	53F	lung cancer	23	-	-	28	1

Total cases expressing HSV   3/17

TABLE 2

Part B - Cultures maintained 9 - 21 days prior to superinfection

Autopsy case	Age and sex	Cause of death	Hrs to explantation	Spontaneous HSV release	Days to spontaneous virus expression	Days to superinfection	Passage at superinfection
A361/80	13M	aortic stenosis	21	-	-	13	1st outgrowth
A370/80	58M	cardiogenic shock	18.5	-	-	15	"
A371/80	49M	adenocarcinoma	20.5	+	12	21	"
A373/80	65F	aortic stenosis	20	+	11	20	"
A375/80	68F	myocardial infarction	8	+	12	12	"
A376/80	61F	heart failure	23	-	-	11	"
A379/80	33F	pneumonia	5	-	-	15	"
A386/80	47F	cerebellar aneurysm	3.5	+	9	9	"
A389/80	70M	myocardial infarction	16	+	10, 11	19	"
A393/80	89F	carcinomatosis	6	+	8 - 10	17	"
A397/80	58F	endometrial adenocarcinoma	8	+	11, 12	14	"
A402/80	57M	abdominal aneurysm	23	+	9	14	"
A413/80	68F	meningitis	15.5	-	-	14	"
A416/80	55M	renal failure	15	-	-	20	"
A419/80	18F	trauma	6.5	-	-	9	"
A424/80	80M	heart failure	16	+	8	17	"
A431/80	79M	hip replacement	6	+	9	14	"
A437/80	68M	cardiorespiratory failure	9	+	9 - 14	21	"
A444/80	84M	cerebrovascular accident	6	+	9 - 17	16	"

Total cases expressing HSV   12/19

TABLE 3   Rescue of HSV from human trigeminal ganglion cultures superinfected with HSV-2 ts mutants

A. Cultures maintained 28-62 days prior to superinfection

Origin[a]	Virus	Incubation temp (°C)	31°C	Progeny virus titer (PFU/ml)	38.5°C
A261 (2) LTG	ts1	31	5.5×10^6		2.0×10^4 (400, 667)[b]
Control BHK C13	ts1	31	1.7×10^6		5.0×10^1
Control fetal brain	ts1	31	8.0×10^4		3.0×10^1
A276 (23) RTG	ts1	38.5	$> 5.0 \times 10^5$	$(2.2 \times 10^3, 1.25 \times 10^4)$	1.0×10^1 (10, 10)
Control BHK C13	ts1	38.5	2.3×10^2		$< 10^1$
Control fetal brain	ts1	38.5	4.0×10^1		$< 10^1$
A274 (19) RTG	ts1	38.5	$> 5.0 \times 10^5$	$(8.3 \times 10^3, 1.25 \times 10^4)$	3.3×10^2 (33, 33)
Control BHK C13	ts1	38.5	6.0×10^1		$< 10^1$
Control fetal brain	ts1	38.5	4.0×10^1		$< 10^1$
A292 (3) LTG	ts1	38.5	$> 5.0 \times 10^5$	$(5 \times 10^4, 6.9 \times 10^2)$	7.0×10^1 (7, 7)
Control BHK C13	ts1	38.5	$< 10^1$		$< 10^1$
Control fetal brain	ts1	38.5	7.3×10^2		$< 10^1$
A292 (4) LTG	ts1	38.5	$> 5.0 \times 10^5$	$(5 \times 10^4, 6.9 \times 10^2)$	$< 10^1$
Control BHK C13	ts1	38.5	$< 10^1$		$< 10^1$
Control fetal brain	ts1	38.5	7.3×10^2		$< 10^1$

TABLE 3

B. Cultures maintained 9-21 days prior to superinfection

A386 (9) RTG	ts1	31	1.5×10^5	3.3×10^3	$(3.3 \times 10^2, 3.3 \times 10^2)$
Control BHK C13	ts1	31	7.1×10^6	$< 10^1$	
Control fetal brain	ts1	31	6.1×10^6	$< 10^1$	
A444 (3) LTG	ts5	31	9.0×10^6	$> 5.0 \times 10^5$	$(5 \times 10^4, ND^c)$
Control BHK C13	ts5	31	6.0×10^5	$< 10^1$	
A444(12) RTG	ts5	31	2.9×10^4	4.3×10^2	$(43, ND)$
Control BHK C13	ts5	31	6.0×10^5	$< 10^1$	

a   Cultures are designated according to the autopsy number from which they were derived. The number in parentheses refers to an individual culture. LTG - left trigeminal ganglion, RTG - right trigeminal ganglion.

b   Numbers in parentheses indicate the ratio of titers of progeny virus from individual ganglion cultures to those from control BHK C13 cultures and fetal brain cultures, respectively. A value of 3 or greater was regarded as evidence of complementation or recombination.

c   ND - not done.

Figure 2. Autoradiographs of A) Kpn I and B) Bam HI digests of
^{32}P-labeled DNA of prototypes HSV-1 17 and HSV-2 HG52, spontaneous
isolates from human trigeminal ganglion cultures and ts+ viruses rescued
following superinfection of a single ganglion culture with HSV-2 ts1.
Isolates 20A, 20B, and 20C are spontaneous isolates from autopsy case
A386/80. Isolates 386-9A, 386-9B, and 386-9E are plaque-purified ts+
viruses recovered after superinfection. (•) Novel fusion or cleavage
products.
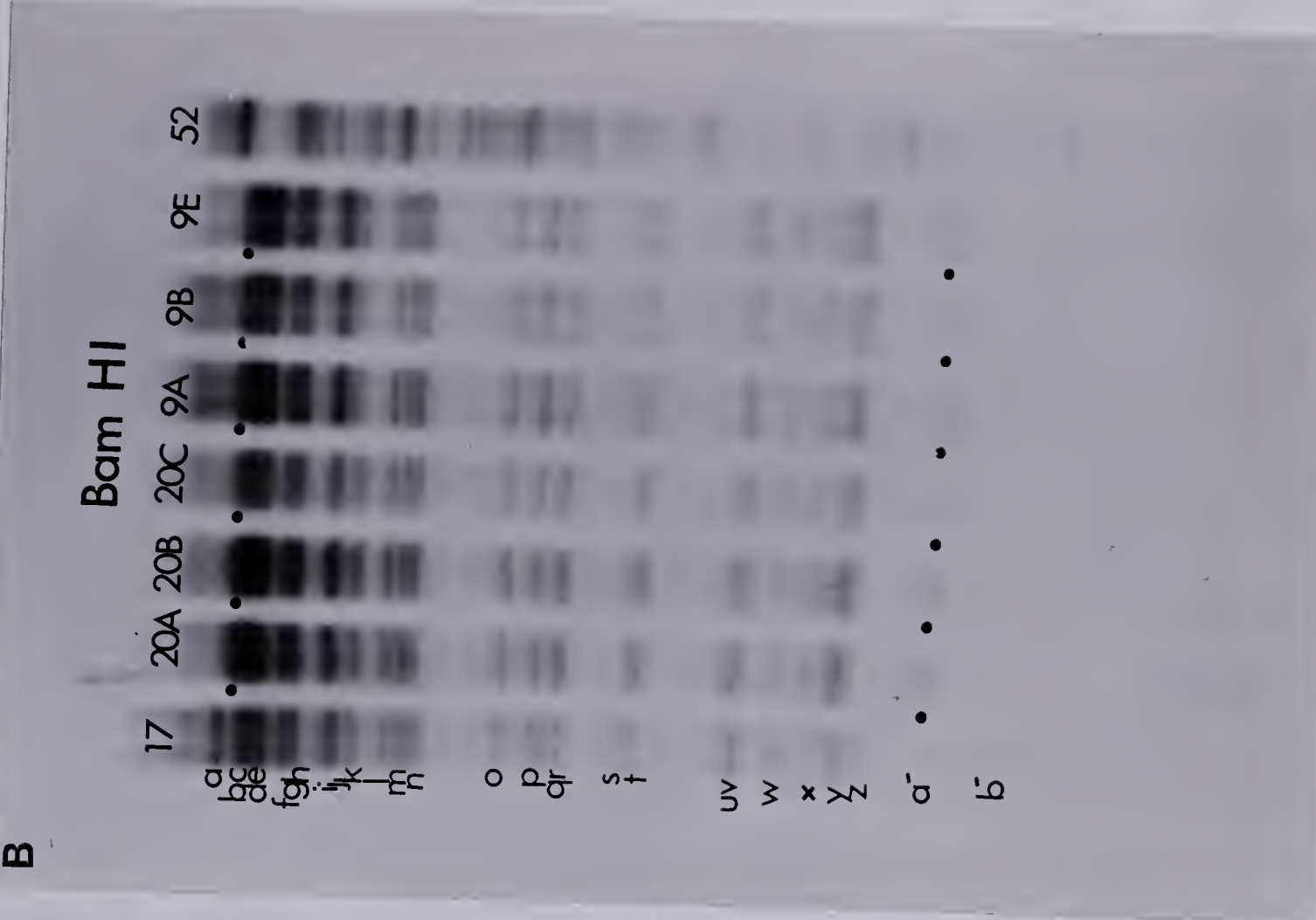
Figure 3. Autoradiographs of A) Kpn I and B) Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17 and HSV-2 HG52 spontaneous isolates from human trigeminal ganglion cultures, and ts+ viruses rescued following superinfection of cultures with HSV-2 ts5. Isolates 28A, 28B, 28C, and 28D are spontaneous isolates from autopsy case A444/80. Isolates 444-3B and 444-3F are plaque-purified ts+ viruses recovered following superinfection of a single culture; 444-12A, 444-12B, and 444-12C are plaque-purified ts+ viruses recovered from a single culture. (•) Novel fusion or cleavage products.

A

Kpn I

17 28A 28B 28C 28D 3B 3F 12A 12B 12C 52
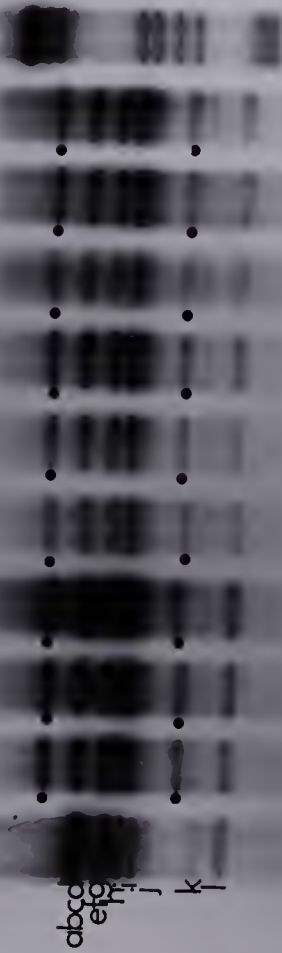
abcd efg hi j k l m no p qr s t u v wx yz a⁻ b⁻

B

Bam HI

17 28A 28B 28C 28D 3B 3F 12A 12B 12C 52

a bcd efg h ij k l m n o p qr s t uv w x yz a⁻ b⁻

CHAPTER V

EFFECT OF ACYCLOVIR ON REACTIVATION OF LATENT HERPES

SIMPLEX VIRUS FROM CULTURED HUMAN TRIGEMINAL GANGLION CELLS

Introduction

The nucleoside analogue acyclovir [(ACV, 9-(2-hydroxyethoxymethyl)

guanine] has been shown to inhibit the replication of HSV-1 and HSV-2 in

vitro (Crumpacker et al., 1979; Elion et al., 1977; Furman et al., 1979;

Schaeffer et al., 1978) and in vivo (Bauer et al., 1979; Cho and Feng,

1980; Kaufman et al., 1978; Park et al., 1979; Pavan-Langston et al.,

1978; Schaeffer et al., 1978), although the drug is not effective in

eradicating latent virus from peripheral nerve ganglia (Field et al.,

1979; Klein et al., 1981; Svennerholm et al., 1981). In the first step

of inhibition, the virus-specified thymidine kinase (TK) phosphorylates

ACV to 5' - monophosphate more efficiently than does cellular TK (Fyfe

et al., 1978). Cellular kinases further phopshorylate ACV monphosphate

to the 5' - triphosphate (Elion et al., 1977; Fyfe et al., 1978). The

acycloguanosine triphosphate form inhibits viral DNA synthesis at the

level of the viral DNA polymerase, which allows the drug to become

incorported into growing DNA chains and thereby effect chain termination

(Furman et al., 1980). The 50% inhibitory dose (ID_{50}) of ACV for HSV-1

has been reported to be 0.15 M ± 0.09 μM and that for HSV-2 to be 1.62

μM + 0.76 μM, as measured by plaque-reduction assays (Crumpacker et al.,

1979).

In the present study in vitro techniques of virus reactivation

have been employed to test the efficacy of ACV in preventing the

spontaneous expression of HSV from cultured human trigeminal ganglion

cells.

Results

HSV was recovered from cultures of human trigeminal ganglia
incubated for 30-45 days in the absence of ACV in 9/20 (45%) of cases
(Table 7). This rate of recovery of HSV from human trigeminal ganglia
derived from randomly selected cadavers is in the 40-60% range
previously reported (Baringer, 1975; Warren et al., 1977). The mean
time elapsed to virus recovery was 14 days from explantation (Table 7).
All viruses isolated were identified as HSV-1 by restriction enzyme
analysis (Figs. 5-13, 17-22, Chapter VII).

In those cultures incubated in the continuous presence of $10 \, \mu M$
ACV for 30 days, 0/23 (0%) of the cultures yielded HSV spontaneously
(Table 8). The difference in recovery rate of HSV in cultures
maintained in the continuous presence of ACV (0%) compared to that
obtained in the absence of ACV (45%) was statistically significant
(χ^2 = 10.51, 1 df, p<0.01).

Cultures from eighteen of the 23 cases incubated in the continuous
presence of ACV for 30 days were released from the drug and maintained
in culture for an additional 15-20 days in medium without ACV (Table 8).
Of these 18, cultures from 3 individuals (cases A079/82, A102/82, and
A211/82) spontaneously expressed HSV following release of ACV. Virus
was recovered from cultures 13, 14 and 20 days following ACV withdrawal.
HSV was also recovered from cultures of trigeminal nerve root derived
from one of these 3 individuals (case A079/82, Chapter IV). The root
and ganglion isolates from case A079/82 were identified by restriction
enzyme analysis as HSV-1 and had identical Bam HI profiles (Chapter IV,
Figure 1).

Discussion

Recently Klein et al., (1981) reported that the continuous presence of acyclovir in concentrations of 5 or 10 μg/ml for 3 to 6 days prevented the in vitro reactivation of HSV from cultures of latently infected murine ganglia. However, when the drug was removed after 3 days, the process of virus reactivation and multiplication resumed. It was concluded that the continuous presence of ACV can prevent further multiplication once the process of virus reactivation is initiated in latently infected ganglia maintained in explant culture.

The above results demonstrated that ACV was able to also prevent the in vitro reactivation of HSV from human trigeminal ganglia obtained from cadavers. Compared to the control group of ganglion cultures incubated without ACV, where HSV was recovered in 9/20 cases, ganglion cultures incubated in the continuous presence of ACV failed to release HSV spontaneously in 23 cases. In cultures released from ACV, virus reactivation occurred in 3/18 cases.

It is not clear why latent HSV was not recovered from more than 3 of 18 cases following removal of ACV. Perhaps, in cultures released from ACV, longer incubation would have increased recovery of HSV. The possibility that continuous incubation with ACV resulted in elimination of latent virus from ganglion cells cannot be discounted. There has been one report of significant reduction of the incidence of ganglionic HSV latency in mice following systemic treatment with ACV (Pavan-Langston et al., 1979) however these data have not been confirmed in other laboratories (Klein et al., 1981).

A more likely explanation for limited recovery of HSV following ACV withdrawal in experiments described here is related to the dilution

of latent foci during repeated passaging of cultured ganglion cells. It
is believed sensory neurons are the source of latent HSV (Cook et al.,
1974; McLennan and Darby, 1980; Stevens, 1975) thus it is conceivable
that latently infected neurons incapable of cell division are lost from
ganglion cultures during prolonged incubation and cell growth.

Alternatively, as suggested by Park et al., (1982), all latent HSV
present in the trigeminal ganglia probably does not reactivate
simultaneously in vitro. In the majority of ganglion cultures which
harbored latent HSV, ACV treatment may have permitted virus
reactivation, though subsequent replication of virus would have been
prevented. In those few cultures with residual latent foci, removal of
ACV after 30 days of incubation may have allowed reactivation of HSV
with progression to a full replicative cycle.

The observation that the continuous presence of ACV inhibited the
recovery of latent HSV from human ganglia in vitro suggested that the
continuous presence of the drug in vivo may also prevent the recurrence
of overt herpesvirus infection. In fact, the level of ACV employed in
the above studies is within the range observed in the plasma of humans
receiving theraputic regimens of ACV (Saral et al., 1981; Whitley et
al., 1982a). Recently, acyclovir has been shown to provide effective
prophylaxis against reactivated herpesvirus infections in patients
receiving bone marrow transplants (Saral et al., 1981). However current
reports of the isolation of acyclovir-resistant mutants following drug
therapy in humans (Burns et al., 1982; Crumpacker et al., 1982) argue
for selective clinical use of this drug.

The above results which illustrated that the continuous presence
of ACV was able to inhibit HSV reactivation, though in several cases

without elimination of latent virus from ganglion cells are in agreement with those obtained in animal model systems (Blyth et al., 1980; Field et al., 1979). Experiments described here provide a useful in vitro model to study the effect of antiviral drugs in inhibiting reactivation of latent herpes simplex virus from human ganglia.

TABLE 4 RECOVERY OF LATENT HSV FROM EXPLANT CULTURES OF HUMAN TRIGEMINAL GANGLIA MAINTAINED IN THE ABSENCE OF ACV

Case number	Age and sex	Cause of death	Hours from death to establishment of explant cultures	Total days in culture	Rescue of HSV[a]	Days in culture to expression of virus
A316/81	44F	multiple sclerosis	9	30	-	-
A337/81	52F	intracerebral hemorrhage	22	31	+	10, 12
A379/81	96F	intracerebral hemorrhage	23	31	+	10, 13
A423/81	21F	congenital heart disease	12	30	-	-
A028/82	61M	cardiovascular arrest	10	31	+	17, 21
A133/82	100M	pancreatic cancer	11	30	+	13, 14
A142/81	60F	motor vehicle accident	10	30	-	-
A170/81	73M	cerebral vascular disease	20	31	-	-
A186/81	72M	lung cancer	20	32	-	-
A187/81	17F	multiple sclerosis	6	32	-	-
A182/81	57M	shock sepsis	13	33	-	-
A298/81	54M	liver cirrhosis	25	30	+	8, 9, 11, 18
A306/81	77M	mycosis fungoides	9	30	+	13, 14, 15, 19, 21
A007/81	24F	cystic fibrosis	6	45	-	-
A014/81	70M	congestive heart failure	21	46	-	-
A035/81	55M	chronic lymphocytic leukemia	8	30	-	-
MS9/81	62F	multiple sclerosis	5	35	+	10
A060/81	63F	pulmonary emboli	7	30	+	9, 12, 16, 23
A081/81	59M	brainstem infarction	18	35	-	-
A132/81	68F	breast cancer	10	30	+	11

Total cases expressing HSV 9/20 (45%)

Range 8-23

Mean 14

a (-) HSV not rescued, (+) HSV rescued.

TABLE 5   RECOVERY OF LATENT HSV FROM EXPLANT CULTURES OF HUMAN TRIGEMINAL GANGLIA MAINTAINED IN THE PRESENCE OF ACV

Case number	Age and sex	Cause of death	Hours from death to establishment of explant cultures	Rescue of HSV in the presence of ACV[a]	Rescue of HSV following release of ACV[a]	Days to virus expression following release of ACV[b]
A019/81	48M	metastatic brain tumor	7	-	N.D.[d]	-
A036/81	66M	lung cancer	7	-	N.D.[d]	-
A092/81	59M	prostate cancer	7	-	N.D.	-
A130/81	35F	breast cancer	21	-	N.D.	-
A137/81	78F	myocardial infarction	18	-	N.D.	-
A207/81	66M	rectal cancer	11	-[c]	-	-
A172/81	82F	pneumonia	10	-	-	-
A315/81	79M	renal failure	23	-	-	-
A321/81	57M	lung cancer	18	-	-	-
A340/81	73F	carcinomatosis	10	-	-	-
A363/81	61M	myocardial infarction	11	-	-	-
A002/82	76F	heart failure	9	-	-	-
A011/82	63F	heart failure	11	-	-	-

TABLE 5 continued

Case number	Age and sex	Cause of death	Hours from death to establishment of explant cultures	Rescue of HSV in the presence of ACV[a]	Rescue of HSV following release of ACV[a]	Days to virus expression following release of ACV[b]
A079/82	78M	cardiopulmonary arrest	16	-[c]	+	13
A102/82	66M	sarcoma	16	-	+	14
A120/82	60M	prostate cancer	9	-	-	-
A113/82	66M	myocardial infarction	17	-	-	-
A134/82	62M	lung cancer	9	-	-	-
A189/82	57M	lung cancer	20	-	-	-
A191/82	36F	breast cancer	8	-	-	-
A211/82	70F	heart failure	23	-	+	20
A212/82	53F	respiratory failure	21	-	-	-
A215/82	82F	myocardial infarction	18	-	-	-

Total cases expressing HSV in the continued presence of ACV-0/23 (0%)
Total cases expressing HSV following release of ACV-3/18 (16.7%)

[a] Cultures were maintained in the presence of 10 μM ACV for 30 days.

[b] Following release of 10 μM ACV, cultures were maintained for an additional 15-20 days.

[c] Latent HSV was recovered from explant cultures of the trigeminal nerve root maintained in the absence of ACV.

[d] N.D. cultures not maintained in the absence of ACV.

CHAPTER VI

RESTRICTION ENZYME ANALYSIS OF MULTIPLE ISOLATES OF LATENT HERPES
SIMPLEX VIRUS RECOVERED FROM INDIVIDUAL HUMAN HOSTS

Introduction

Restriction enzyme analysis of HSV DNAs has demonstrated that the
overall electrophoretic profiles of DNA fragments allow differentiation
between strains of HSV-1 and HSV-2. Furthermore minor variations in
cleavage patterns enable the identification of strains within a serotype
(Hayward et al., 1975a; Skare et al., 1975).

Previous studies employing restriction endonuclease digestion of
DNAs of latent HSV-1 recovered from human cadavers have shown that in
the majority of cases, isolates from the ganglia of different
individuals can be distinguished from one another. However, virus
isolates derived from the trigeminal, superior cervical and vagus
ganglia, or from the left and right ganglia of the same individual, or
multiple isolates derived from different explant cultures of a single
ganglion were indistinguishable by this criterion (Lonsdale et al.,
1979, 1980).

It has been demonstrated that individuals can harbor latent HSV-1
in the trigeminal ganglia and HSV-2 in the sacral ganglia simultaneously
(Forghani et al., 1977). In addition, a mixture of HSV-1 and HSV-2 has
been recovered from the same genital sites of individual patients on
repeated occasions (Fife et al., 1983). This observation illustrated
that HSV-1 and HSV-2 can cause concomitant overt infection at the
periphery and simultaneous latent infection within one or more ganglia
which innervate the site of recurrence. Within individual hosts, the

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recovery of non-identical strains of HSV-2 from genital lesions and genetically different strains of HSV-1 from brain biopsy specimens and orolabial lesions has been reported (Buchman et al., 1979; Whitley et al., 1982). Thus individuals can become overtly infected with more than one strain within an HSV serotype.

The above data suggested the possibility that within individual human hosts multiple strains of latent HSV could be recovered from the same ganglia or from different ganglia which innervate proximal peripheral sites. The hypothesis of Lonsdale et al. (1979, 1980) that a single virus strain infects each individually initially and virus descended from this event subsequently becomes latent in different cells of the same ganglion as well as in different ganglia, was based, in part, on the analysis of two or more virus isolates obtained from 9 individuals. To re-examine the above hypothesis, restriction endonuclease analysis was employed to compare the DNAs of three or more independent isolates of latent HSV recovered from the same or different sites of 20 individuals.

Results

A. Origin of latent HSV isolates recovered from human cadavers

The origin of 115 isolates recovered from 20 cadavers is shown in Table 9. Three or more isolates were recovered from separate explant cultures derived from the ganglia of each individual. Virus was isolated from both the left and right trigeminal ganglia in 13 individuals and from either the left or right ganglion from 7 individuals. In one case (A060/81) virus was isolated from the left and

right trigeminal nerve roots as well as from both trigeminal ganglia.
In another case, (A401/82) virus was recovered from the left vagus
ganglion and the right trigeminal ganglion.

Virus specific cytopathic effect was observed between 8 and 30 days
(mean 12.9 days) following establishment of in vitro cultures.

Thirteen isolates were recovered from separate explant cultures
derived from one individual, 12 isolates were recovered from another
individual, and 10 isolates were obtained from another individual.
Eight isolates were obtained from 2 individuals, 7 isolates were
recovered from 2 individuals and 6 isolates were recovered from 2
individuals. In 5 cases, 4 isolates were obtained and in 6 cases 3
isolates were recovered (Table 9).

B. Restriction enzyme analysis of HSV DNAs

Isolates were individually digested with Hind III, Kpn I and Bam HI
restriction enzymes and the fragment profiles compared to HSV-1 Glasgow
strain 17 and in most cases, to HSV-2 strain HG52, (Figs. 2, 3, 5-15,
17-22). Each isolate exhibited a restriction enzyme profile
characteristic of HSV-1 with all enzymes used. None of the isolate
profiles resembled that of the HSV-2 prototype strain, nor did any
isolate appear to be an HSV-1 x HSV-2 intertypic recombinant. A
diagrammatic representation of the HSV-1 genome and Hind III, Kpn I and
Bam HI restriction endonuclease maps for HSV-1 strain 17 are shown in
Fig. 4. Hind III cleaves the HSV-1 17 genome at 11 sites, Kpn I at 29
sites and Bam HI at 41 sites.

Within the typical HSV-1 restriction profiles, individual isolates
exhibited changes in fragment number and mobility. The changes observed

in restriction patterns of the isolates compared to HSV-1 strain 17 were classified into two major categories (Buchman et al., 1980; Lonsdale et al., 1979, 1980): (i) The loss or gain of restriction endonuclease sites resulting in the generation of new fragments by fusion or additional cleavage and (ii) variation in electrophoretic mobility of certain fragments due to the insertion or deletion of specific sequences contained within those fragments. Fragments which exhibit variable mobility have been shown to originate from the long and short repeat termini (TR_L and TR_S), the internal joint (IR_L/IR_S), unique-repeat junctions, and in a very small number of cases, from unique regions of the HSV genome (Buchman et al., 1980; Lonsdale et al., 1980).

1. Variations due to loss or gain of restriction endonuclease sites

Figures 2, 3, 5-15, and 17-22 show restriction enzyme profiles of multiple strains of latent HSV-1 recovered from 20 unselected human cadavers. The fragment nomenclature conforms to that of Wilkie (1976), Wilkie et al. (1978) and Davison and Wilkie (1981) as shown in Fig. 4.

Comparison of the DNA of virus isolates with prototype HSV-1 strain 17 disclosed bands which had no counterpart in the reference strain, as well as prototype strain bands missing from the profiles of experimental strains. Where a new band was larger than two missing reference strain bands, it was usually found that the molecular weight of the novel band was approximately equal to the sum of the molecular weights of the two missing bands. Since the missing fragments were always contiguous on the physical maps of HSV-1 17, it was evident that the novel band originated from the loss of a restriction enzyme cleavage site between the contiguous fragments. In the case where a standard strain band was

missing and two new bands appeared which had a total molecular weight equal to that of the missing band, it was deduced that the experimental strain contained a restriction site within the standard strain fragment.

In many instances it was observed that one standard band strain was missing from the experimental strain, but that no additional bands or only one new band appeared. However, one of the other standard strain bands in the autoradiogram had an increased intensity indicating that the new band(s) comigrated with a standard band. This observation was confirmed, in one series of strains, by Southern blotting data (Figure 16). If ambiguities arose, reference to published restriction maps of other HSV-1 strains (Locker and Frenkel, 1979) often facilitated analysis. It is to be recognized that ultimate proof of the origin of novel fusion or cleavage products can only be obtained by hybridization data.

Table 10 gives a precise description of variable restriction enzyme cleavage sites observed in the DNA of isolates recovered from human cadavers. Nomenclature of variable sites is based on that described previously (Chaney et al., 1983). Among viral DNAs analyzed, two variable sites were found upon Hind III digestion. Seven variable sites were observed with Kpn I and Bam HI restriction enzymes.

In Table 11, variable restriction enzyme cleavage sites of isolates derived from each individual, and in select cases, of individual isolates are compared. In 18 of 20 cases, all isolates derived from a single host were identical with respect to the presence or absence of the specified restriction sites. The exceptions were found in isolates 31A-M (case A060/81) and isolates 49A-F (case A401/82). Additionally, isolates derived from each of 18 individuals could be distinguished by

this criterion. These data conformed to those reported in previous studies (Lonsdale, 1979, 1980).

One anomaly was noted. The Bam HI digestion pattern of the DNA of isolate 37B revealed the presence of an additional fragment of approximately 2.8 Md (Figure 18C). This fragment, which migrated between Bam HI-n and Bam HI-o, was not observed in the DNA profiles of the other nine viruses isolated from the same individual. The appearance of this novel band was not accompanied by the loss or gain of any other fragment, and therefore its origin is difficult to explain. It is possible a discrete block of either host or viral DNA sequences was inserted into the genome of isolate 37B. However, the appearance of a novel fragment implies such an insert was bounded by Bam HI recognition sites. Alternatively, isolate 37B may have consisted of a mixed population of viral DNAs, the majority of which had a Bam HI profile identical to that of the other isolates derived from this individual, and a minority population in which a spontaneous mutation resulted in the acquisition of a new Bam HI cleavage site. Additional cleavage products or loss of fragments may have resulted but were not detectable.

In contrast to the above findings, where HSV isolates derived from single hosts were genetically identical, the DNAs of HSV isolates from two individuals differed among themselves in the number and location of restriction sites with at least two of three enzymes used.

Analysis of isolates 31A-M

In one cadaver, 13 HSV isolates were recovered from parallel cultures of explanted tissue from the trigeminal ganglia and nerve roots. Isolates 31A-M were derived from the left (31A) and right (31B)

trigeminal nerve roots and left (31D, E, F, G, H, I) and right (31C, J, K, L, M) trigeminal ganglia of a 63 year old female whose cause of death was pulmonary emboli (Table 9). Although all isolates had identical Hind III restriction enzyme profiles, Bam HI digests of DNA from virus isolates 31A to M revealed the presence of at least four distinguishable profiles (Fig. 13C). Isolates 31A, D, and F had similar profiles to one another, as did isolates 31B, E, I, K, L and M; isolates 31H and J; and isolates 31C and G. Kpn I digestion showed two different profiles (Fig. 13A and B). In view of past analysis of many human HSV clinical isolates which show that epidemiologically related isolates have identical restriction enzyme patterns (Buchman et al., 1978, 1980; Linneman et al., 1978; Lonsdale et al., 1979, 1980; Roizman and Tognon, 1982), these results immediately indicated that the presence of multiple strains of latent HSV-1 within the trigeminal nerve complex of one individual.

In order to ascertain whether each virus isolate consisted of homogeneous population or mixtures of different strains in equal or unequal proportions, two cycles of plaque purification were performed on four isolates which illustrated three different Bam HI restriction profiles. Nineteen subclones derived from isolates 31A, 31C, 31G and 31K exhibited six differentiable Bam HI profiles and two distinct Kpn I profiles (Table 12, Figs. 14A and B, 15A and B). Subclones of isolate 31A had identical Bam HI profiles but segregated into two strains upon Kpn I digestion. Isolate 31C consisted of a mixture of strains showing four distinct Bam HI profiles and two Kpn I profiles. Subclones of 31G exhibited three distinct Bam HI patterns and two different Kpn I profiles. Subclones of 31K had two different Bam HI profiles and two

different Kpn I profiles, however subclone K5 appeared to be a mixture of strains exhibiting two different Bam HI profiles.

Where identity of subclones was established with one enzyme, as with Bam HI digests of strains A1 and A3, digestion with an additional enzyme, Kpn I, unambiguously differentiated between the two strains. Thus, the combined data from Kpn I and Bam HI restriction enzyme profiles indicated the existence of eight separable strains among isolates 31A, 31C, 31G and 31K. They were represented by strains (1) C1 and K1; (2) A1, A2, A5, G1, G3, G5; (3) A3; (4) C4 and G2; (5) C2 and K4; (6) K2 and K3; (7) G4; and (8) C3 and C5. Analysis of all subclones indicated multiple strains were recoverable from each ganglion and from the left trigeminal nerve root and therefore that individual strains were not associated with distinct neuroanatomical sites.

In order to confirm several of the restriction site changes and strain classifications referred to above, Southern blot hybridization was performed on several subclones. The DNAs of selected strains were digested with Bam HI and Kpn I and transferred to nitrocellulose filters by the method of Southern (1975) as described in Materials and Methods. Probes of ^{32}P-labeled Bgl II fragments k or jl derived from HSV-1 strain KOS were used for hybridization.

Figure 16 shows the hybridization of Bgl II-k to Bam HI digests of several subclones of 31C, G, and K. Bgl II-k (located at map position 0.10 - 0.16, refer to Fig. 4) is contained with fragments Bam HI-c (position 0.80 - 1.40) and Bam HI-a (position 1.40 - 2.20). As predicted, hybridization of Bgl II-k to HSV-1 17 Bam HI-a and Bam HI-c was observed (Fig. 16A). A similar pattern was observed in subclones K1, C1, C3 which confirmed that the Bam HI-a standard fragment was

intact in these strains. Strain K5, a presumed mixture of strains with Bam HI patterns II and IV (Table 12) showed hybridization of Bgl II-k to a low molecular weight cleavage product of Bam HI-a (0.8 Md) as well as to the 7.2 Md cleavage product (consistent of pattern II) and to a 2.2 Md fragment characteristic of the pattern IV profile. In strain C2, Bgl II-k hybridized to the 2.2 Md Bam HI-a cleavage product, consistent with a Bam HI pattern IV profile. Strains C4 and G2 also exhibited fragment hybridization which correlated with a Bam HI pattern III profile. In strain G4 (Bam pattern V) Bgl II-k hybridized to 0.8 Md Bam HI-a cleavage product. As expected, Bam HI-c hybridized to Bgl II-k in all strains. Therefore, in all subclones examined the presence or absence of cleavage sites within the standard strain fragment Bam HI-a as shown by hybridization data was completely consistent with the analysis of Bam HI restriction enzyme profiles.

Hybridization of Bgl II fragment jl to restriction enzyme digests of various subclones did not clearly distinguish one strain from another (Fig. 16B and C). Bgl II-jl is an internal joint fragment present in the I_{SL} arrangement of the HSV-1 genome. In the prototype orientation of the HSV-1 17 genome, fragment j is located at the terminal repeat of the L segment (TR_L) and extends into the U_L region (map position 0.00 - 0.06) and fragment l is located at the terminus of S (TR_S) and extends into U_S (map position 0.96 - 1.0). Restriction site changes in the TR_L, TR_S or IR_L/IR_S regions of the genome were not used to separate strains within isolates 31A-M, however hybridization to standard strain fragments located in these regions was observed. In addition, the existence of cleavage sites within standard strain Kpn I fragments b and g was confirmed by hybridization of Bgl II-jl to the 6.2/1.8 Md and

5.2/1.8 Md cleavage products of these fragments, respectively (Fig. 16C, Table 10).

Analysis of isolates 49A-F

Isolates 49A-F were obtained from a 65 year old male who died from a cardiac arrest. The restriction enzyme patterns of five virus isolates recovered from the right trigeminal ganglion (49B-F) were identical but differed from those of a single isolate from the vagus ganglion (49A). With Hind III, Kpn I and Bam HI, there were differences in the presence or absence of restriction sites between isolate 49A and isolates 49B-F (Fig. 22, Table 11).

The Hind III restriction enzyme digestion pattern of strain 49A was identical to that of prototype HSV-1 17. Isolates 49B-F were characterized by the loss of Hind III-h (7.0 Md) and Hind III-o (1.9 Md) and the appearance of a novel fusion fragment Hind III-h/o (8.9 Md) which migrated between Hind III-f and Hind III-g. The Kpn I digestion patterns of strains 49B-F were identical, whereas strain 49A was characterized by the loss of fragment m (3.1 Md) and the appearance of a fragment which migrated faster than fragment qr (2.6 Md) and one of 0.5 Md which migrated between Kpn I-a' and Kpn I-b' visualized on a 3.5% polyacrylamide gel. The Bam HI restriction patterns of strains 49B-F were identical and were characterized by the presence of a high molecular weight Bam HI-d/h fusion fragment. Strain 49A also contained this fragment, but had an additional cleavage site within Bam HI-a.

In this case, as in one discussed above for isolates 31A-M, restriction site changes occured at multiple genomic loci. In contrast to the above situation where multiple strains were recoverable within the same neuroanatomical site, among isolates strain 49A-F, a single

strain was unique to the vagus ganglion and another to the trigeminal ganglion. However, since plaque-purified subclones were not obtained from this individual, the possibility that strain 49A comprised a minority population among isolates obtained from the trigeminal ganglion can not be rigorously excluded.

2. Variations due to changes in the electrophoretic mobility of various restriction endonuclease fragments.

When isolates from different individuals or from a single individual were compared variations in the electrophoretic mobility of certain restriction enzyme fragments were observed. The criterion used to establish alterations in fragment mobility due to the insertion or deletion of a number of DNA base pairs was the absence of new cleavage or fusion products which could account for the mobility shift.

The restriction fragments which have been observed to exhibit alterations in mobility originate from the following defined areas of the HSV-1 genome: (1) the restriction fragments of the long and short repeat termini (TR_L, TR_S) and the internal joint fragment (IR_L/IR_S), (2) the restriction fragments spanning the unique-repeat junctions of both the long and short segments of the genome and (3) Bam HI-z (Lonsdale et al., 1980). With the three enzymes used in this study the standard strain (HSV-1 17) fragments referred to above include Hind III-m; Kpn I-r, b, g, j, k, a and e; and Bam HI-s, e, b, q, y, n, z and x (refer to Fig. 4). Additionally, in many of the experimental strains, novel fragments resulting from the internal cleavage of standard strain fragments exhibited expected mobility variations.

Mobility variations in certain fragments were observed in the

restriction profiles of virtually all strains examined, however only select examples of variations between isolates from different individuals as well as among isolates from the same individual will be discussed. As in past studies (Lonsdale et al., 1979, 1980) variation in fragment mobility in isolates derived from a single host was less notable than that observed in isolates from unrelated hosts.

Figs. 6B and 6C show Kpn I and Bam HI restriction profiles of isolates 18A-C, 21A-D and 22A-D; each set of isolates was derived from a different individual. In the Kpn I profile, variability of fragments k and a fragment which co-migrated with HSV-1 17 Kpn I-s was observed and was more marked between isolates from different individuals than among isolates from the same individual. A similar observation was made for new cleavage fragments which co-migrated with HSV-1 17 Kpn I-u and Kpn I-h. The Bam HI profiles of the same isolates show variability in fragments k, e, n, x, y and z that was more extensive between isolates from different hosts than among those derived from the same host. Isolates 30A-C obtained from one host and isolates 43A-D obtained from another host also exhibited variation in mobility of certain restriction fragments that was greater in isolates between the two individuals than that observed in isolates within either individual (Fig. 12C). Bam HI-k, n, s and x were most notable in this respect. Enhanced alteration in fragment mobilities observed between unrelated isolates relative to those seen among related isolates supported the observation discussed above that in 2 of 20 individuals, multiple strains of latent HSV were isolated. Specifically, the variation in fragments Hind III-m; Bam HI-k, y, Kpn I-j, k, and novel fragments co-migrating with Kpn I-s, u, and h between isolate 49A and isolates 49B-F (Fig. 22) was as notable

as that exhibited between isolates derived from different hosts.

Within many individual hosts, alteration in electrophoretic mobility of several fragments was also observed. For example, the Hind III profiles of isolates 22A-D showed variation in fragment m (Fig. 6A). A novel cleavage product of Kpn I-g which migrated between Kpn I-j and Kpn I-k in isolates 38A-D also displayed mobility variations (Fig. 19). Among isolates 36A-H, Bam HI-k exhibited marked mobility heterogeneity as shown by the "fuzzy" or "stepladder" appearance of this fragment in agarose gels (Fig. 17).

Mobility variations within the reiterated regions and unique-repeat junctions of the HSV-1 S component were notable in almost all cases, and in one particular instance posed interpretational difficulties. Fig. 3B (Chapter V) shows the Bam HI profiles of isolates 28A-D. The observed variability in fragments Bam HI-x, y and z appeared greater than observed elsewhere but could have been due to insertions and deletions in these fragments as discussed above. Alternatively, variability could have been due to new cleavage sites within the region spanning these fragments, since the sum of the molecular weights of fragments which migrated between Bam HI-w and Bam HI-a' had a total molecular weight equal to that of Bam HI-x, y and z.

Discussion

Previous studies employing restriction endonuclease analysis have established molecular identity between herpes virus strains isolated from the lesions of epidemiologically related individuals (Buchman, 1978; Linnemann et al., 1978) but have also shown virus strains obtained from overt infections are genetically dissimilar between unrelated hosts or in cases where an individual was infected with more than one strain

of HSV, either successively or concurrently (Buchman et al., 1979; Fife et al., 1983; Whitley et al., 1982b). However, when latent HSV obtained from human cadavers was reactivated in vitro, molecular analysis of isolates revealed the presence of a single and unique virus strain within the ganglia of each individual (Lonsdale et al., 1979, 1980).

In the present study, 115 isolates derived from the trigeminal and vagus ganglia and trigeminal nerve roots of 20 individuals were compared by restriction enzyme analysis to determine if multiple strains of latent HSV could be isolated within individual human hosts. In addition, the extent of molecular variability between isolates from epidemiologically unrelated individuals was compared.

One significant observation resulting from the above analysis was that all isolates were identified as HSV-1. Though HSV-1 is the predominant serotype associated with orolabial lesions and rare instances of herpes encephalitis, and HSV-2 with genital lesions and neonatal infections, (Dowdle et al., 1967; Nahmias and Roizman, 1973) both serotypes have been isolated from the oral and genital sites of humans (Chaney et al., 1983; Fife et al., 1983; Nahmias et al., 1981). However, isolation of HSV-2 from the human oropharynx has been reported to be rare (Nahmias et al., 1981) and so, in studies reported here, it was not unexpected that latent HSV-2 was not recovered from ganglia which innervate this anatomical region.

Analysis of three or more independent isolates obtained from each of 20 individuals revealed the existence of 16 variable cleavage sites with three restriction enzymes used. For HSV-1 17 a total of 81 cleavage sites exist for Hind III, Kpn I, and Bam HI, restriction enzymes. In the majority of experimental strains analysed in this

study, the presence or absence of low molecular weight fragments Kpn I-c' and Bam HI-c' - m' was not determined. Therefore, in most cases a total of 69 sites was surveyed. The map locations of variable sites are summarized in Fig. 23.

The present study, which considered molecular relationships between latent herpes viruses isolated from the same hosts as well as those recovered from epidemiologically unrelated individuals, required a clear and precise definition of a virus strain. Previous reports concerned with the application of restriction enzyme analysis to molecular epidemiologic problems have differred in this respect. Lonsdale et al. (1980), in his study of 44 ganglion isolates from 21 individuals, used as a basis for analysis only those differences resulting from loss or gain of 2 or more restriction sites with nine enzymes without regard to variation in electrophoretic mobility of specific restriction fragments. Buchman et al. (1980) in their review of five case studies concerned with transmission of HSV-1 and HSV-2 and recurrent infection with HSV-2, used as criteria for genetic non-identity, the loss or gain of single restriction sites with two to five enzymes or a change of at least ten percent in the electrophoretic mobility of a given fragment band with respect to the comparable band in other profiles. In the report of Chaney et al. (1983) 29 HSV-1 strains were differentiated solely on the basis of a single variable restriction site with eight enzymes used, however the differentiation of 39 strains of HSV-2 was based on a combination of variable length fragment data and variable restriction sites with five enzymes.

In the above study, the loss or gain of a single restriction site among three enzymes, which could be accounted for by the appearance of

novel fusion or cleavage products was used as the sole criterion of genetic non-identity, and hence strain differentiation. Since the molecular weights of variable fragments were not measured, alteration in electrophoretic migration of fragments was not used as parameter to establish genetic dissimilarity. In addition, the use of the term strain referred only to a unique restriction enzyme profile and was not used to imply antigenic differences.

By the criterion of number and location of restriction enzyme cleavage sites utilized here, the genetic identity of isolates from the left and right trigeminal ganglia or of multiple isolates from a single ganglion within 18 of 20 individuals was established. It was also shown that isolates from these individuals could be distinguished from each other purely on the basis of variable restriction site combinations.

The majority of individuals sampled in this study harbored only one strain of latent HSV-1 despite probable exposures to additional strains. However, in two individuals multiple strains of latent HSV-1 were recovered from the same or different neuroanatomical sites.

Recovery of non-identical strains of latent HSV-1 within single hosts suggested multiple strains arose by exogenous reinfection or by concurrent infection with more than one of HSV. Due to the lack of detailed serological and clinical information from the patients cited in this study, these situations could not be differentiated.

Alternatively, the probability that multiple virus isolates of HSV arose by genetic alteration of one initial strain in vivo or cultivated in vitro is small. Spontaneous mutations resulting in the loss or gain of restriction sites have not been attributed to repeated passage of

virus in culture nor to prolonged residence within the host (Buchman et al., 1980; Lonsdale et al., 1980). In fact, extensive studies involving analysis of plaque-purified clones of stock strains have failed to reveal a single instance of a change in the number and location of restriction endonuclease cleavage sites by in vitro propagation (Roizman and Tognon, 1982). The virus isolates analyzed in the above study were passaged only one time in culture at low multiplicities of infection.

The existence of genetically dissimilar strains within individual hosts which exhibit multiple nucleotide changes mapping at different sites in the HSV genome has been demonstrated; the likelihood such changes arose spontaneously is virtually nil. In fact, the extent of variability used to define genetic non-identity of virus isolates may be much greater than observed, since only a small fraction of nucleotides has been analyzed by restriction enzyme digestion.

However, the possibility that some of the strains among isolates 31A-M arose by intratypic recombination between any pair of strains initially present either at the site of peripheral infection or latency within the host, or during the course of in vitro culture can not be excluded. Further analysis of the virus isolates recovered from this individual may clarify the genetic relationship, if any, between these strains. However, it is recognized that the generation of recombinants requires, a priori, the co-existence of at least two non-identical parental strains of HSV.

An additional aspect of the above study warrants emphasis. The purification of genetically dissimilar subclones from isolates 31A-M underscores the need for plaque-purification of strains if the homogeneity of a given virus population is not readily apparent. In the

previous studies of Lonsdale et al. (1979, 1980), which established molecular identity of virus isolates within single hosts, the majority of test stocks were not plaque-purified and assumed to be free of contamination with a second HSV-1 strain. Here, the existence of different virus strains, among both heterogeneous and seemingly homogeneous populations was demonstrated. Isolates 31A and 31K appeared to have pure and distinguishable Bam HI profiles (Fig. 13C), however subclones segregated into several distinct patterns. This was not unexpected in the cases of isolates 31C and 31G as their Kpn I and Bam HI restriction profiles (Fig. 13B and C) were indicative of a mixed population of virus strains. Thus, further epidemiologic studies concerned with the genetic relatedness of herpesvirus strains from the same or different individuals must consider the possibility that the presence of a majority virus component may mask identification of a minority component within the population.

The above findings have other important implications. Recently, the isolation in humans of herpesviruses resistant to antiviral drugs has been reported (Burns et al., 1982; Crumpacker et al., 1982). In the case of an individual with a mixed herpesvirus infection, the resistance of a single strain may confer a phenotypic drug resistance upon a population consisting of both susceptible and resistant strains. Secondly, the ability of an individual human host to harbor multiple strains of latent HSV within single or multiple ganglia suggests, if even in only a minority of individuals, that the immune response elicited against an initial herpesvirus infection may not be protective against subsequent exposures to strains of the same or different serotypes. It thus appears that immune mechanisms operating in the

human host cannot be directly compared to those in experimental animals where latent ganglionic infection established with one strain of HSV-1 prevented colonization of the ganglion by superinfecting genetically distinguishable strains of HSV-1. (Centifanto-Fitzgerald et al., 1982). These points warrant consideration in regard to both antiviral and future vaccine therapies.

Lastly, the data presented above are relevant to hypotheses explaining the origin and mechanisms of molecular variability of herpes-viruses. In this study and those of others (Buchman et al., 1980; Chaney et al., 1983; Lonsdale et al., 1979, 1980) the extent of variability of HSV DNAs has been assessed by restriction enzyme analysis. By definition, a technique which surveys only a minority of nucleotides is likely to underestimate the magnitude of genomic flexibility. Nevertheless, it appears as if variability is confined to only a fraction of restriction sites, the majority of which are stable.

Though insufficient data are available to substantiate the idea that nucleotide changes, insertions, or deletions occur at non-essential sites in the genome, logic dictates this to be the case. For example, in genes which have been sequenced, such as the immediate-early polypeptides (V_{mw} 12, V_{mw} 68 and V_{mw} 175), variable numbers of tandemly repeated sequences occur in introns in the case of V_{mw} 175, or in non-coding regions of the polypeptides V_{mw} 12 and V_{mw} 68 (Murchie and McGeogh, 1982).

Recently, the complete nucleotide sequence of the HSV-2 strain 333 thymidine Kinase (TK) gene was obtained and compared to that of the HSV-1 strain MP (McKnight, 1980) and HSV-1 strain CL 101 (Wagner et al., 1981) TK genes in order to assess the degree of intra- and intertypic

variation for a single viral gene (Swain and Galloway, 1983). It was found that the nucleotides encoding the structural gene varied 1.7% between the two HSV-1 strains and 19% between HSV-1 and HSV-2. Approximately half of the nucleotide changes resulted in amino acid substitutions. Between the two HSV-1 strains, the amino acid sequence of the TK protein differed 1.9%; between HSV-1 and HSV-2, 27% of the amino acid residues differed. Conservation of the DNA sequences in the 5' non-coding region of the gene was more marked than that within the coding region, however in the 3' non-coding region, homology was least extensive. Swain and Galloway (1983) concluded that a series of base changes was the basis of genetic variability between the TK genes of HSV-1 and HSV-2. No evidence was obtained for major chromosomal rearrangements in this area of the genome, in fact, it was found that variation was conservative, with transitions and transversions occurring at approximately equal frequencies and more commonly than insertions and deletions of single nucleotides. However, it was suggested that constraints on the TK gene may not be found in other classes of viral proteins.

As the DNA sequences of more HSV-1 genes become available, the extent to which the virus can tolerate genetic variability without loss of viability will be revealed. It is assumed single base changes in the third position of certain codons which would not alter the amino acid sequence of a given polypeptide (wobble) could be maintained. Similarly missense mutations which would not disrupt polypeptide function could be a source of genetic diversity. Insertions, deletions or point mutations occurring in regions non-obligatory for accurate and quantitative gene transcription could also contribute to molecular variation.

Roizman and co-workers (Buchman et al., 1980) have attributed the genetic variability of herpes simplex viruses to the accumulation and persistence of spontaneous, though rare mutations which have occurred over the millenia of the existence of HSV. Implied in this hypothesis is that such variants are not displaced from the human population. Due to the ability of HSV to remain latent over the lifetime of the host, and the mode of transmission of the virus, it was theorized that viral variants cluster among those individuals in close contact.

However, these workers have also stated unequivocally that genotypic differences between virus isolates are stable in vitro and in vivo. To the contrary, Lonsdale et al. (1980) reported that mutational events resulting in the loss or gain of restriction sites, though rare, do occur among clonally related isolates at the rate of one site difference with nine enzymes in one in 15 to one in 30 isolates. Though this appears to be a relatively high rate of spontaneous mutation it is difficult to reconcile these observations with those of Buchman et al. (1980). A more tenable hypothesis of the origin of HSV genomic variability is that spontaneous mutations do occasionally occur during residence in the host, and that minority populations containing such mutations can be transmitted to other hosts if the genetic lesion is one that is non-lethal. It remains to be determined if the occurrence, though likely infrequent, of multiple rather than unique latent strains of HSV within individual human hosts has modified the epidemiology or even the evolution of the herpes simplex viruses.

TABLE 6   ORIGIN OF LATENT HERPES SIMPLEX VIRUS ISOLATES RECOVERED FROM HUMAN CADAVERS

Case number	Age and sex	Cause of death	Isolate designation	Days in culture to expression of virus	Neuro-anatomical site
A303/79	65M	colon cancer	8A	20	LTG[a]
			8B	30	LTG
			8C	30	LTG
A375/80	68F	myocardial infarction	18A	12	LTG
			18B	12	LTG
			18C	12	LTG
A386/80	47F	cerebellar aneurysm	20A	9	LTG
			20B	9	LTG
			20C	9	RTG[b]
A393/80	89F	carcinomatosis	21A	8	RTG
			21B	8	RTG
			21C	9	RTG
			21D	10	RTG
A389/80	70M	myocardial infarction	22A	10	RTG
			22B	10	RTG
			22C	10	LTG
			22D	14	LTG
A397/80	58F	endometrial cancer	23A	11	RTG
			23B	11	LTG
			23C	12	LTG
A402/80	53M	abdominal aneurysm	24A	9	RTG
			24B	9	RTG
			24C	9	RTG
			24D	11	RTG
			24E	11	LTG
			24F	14	RTG
A431/80	79M	hip replacement surgery	26A	9	LTG
			26B	9	LTG
			26C	9	LTG
			26D	9	LTG
			26E	11	LTG
			26F	13	LTG
A424/80	80M	cardiac failure	25A	8	RTG
			25B	8	RTG
			25C	8	RTG
			25D	8	RTG
			25E	8	RTG
			25F	8	LTG
			25G	9	LTG
			25H	9	LTG
			25I	9	RTG
			25J	11	RTG
			25K	12	RTG
			25L	13	LTG

TABLE 9 continued

A437/80	68M	respiratory failure	27A	9	RTG
			27B	9	LTG
			27C	10	RTG
			27D	10	RTG
			27E	11	LTG
			27F	12	LTG
			27G	12	LTG
			27H	14	LTG
A444/80	84M	brainstem infarction	28A	9	LTG
			28B	9	LTG
			28C	14	LTG
			28D	18	RTG
MS1/81	62F	multiple sclerosis	30A	10	RTG
			30B	10	RTG
			30C	10	RTG$_c$
A060/81	63F	pulmonary emboli	31A	9	LTR$_d$
			31B	9	RTR
			31C	9	RTG
			31D	9	LTG
			31E	12	LTG
			31F	12	LTG
			31G	12	LTG
			31H	12	LTG
			31I	12	LTG
			31J	12	RTG
			31K	12	RTG
			31L	16	RTG
			31M	23	RTG
A298/81	54M	liver cirrhosis	36A	9	RTG
			36B	8	LTG
			36C	8	LTG
			36D	9	LTG
			36E	9	LTG
			36F	11	RTG
			36G	11	RTG
			36H	18	LTG
A306/81	77M	mycosis fungoides	37A	14	LTG
			A37B	13	RTG
			A37C	13	RTG
			A37D	14	RTG
			A37E	14	RTG
			A37F	15	RTG
			A37G	19	LTG
			A37H	19	RTG
			A37I	21	RTG
			A37J	30	LTG

TABLE 9 continued

A337/81	52F	intracerebral hemorrhage	38A	10	LTG
			38B	10	LTG
			38C	10	LTG
			38D	12	LTG
A379/81	96F	intracerebral hemorrhage	40A	10	LTG
			40B	13	LTG
			40C	13	LTG
A028/82	61M	cardiovascular arrest	41A	17	LTG
			41B	17	LTG
			41C	17	LTG
			41D	17	LTG
			41E	17	LTG
			41F	21	RTG
			41G	21	RTG
A133/82	100M	pancreatic cancer	43A	14	RTG
			43B	13	RTG
			43C	14	LTG
			43D	13	LTG
A401/82	65M	cardiac arrest	49A	12	LVG[e]
			49B	22	RTG
			49C	25	RTG
			49D	25	RTG
			49E	25	RTG
			49F	25	RTG

[a] LTG - left trigeminal ganglion

[b] RTG - right trigeminal ganglion

[c] LTR - left trigeminal nerve root

[d] RTR - right trigeminal nerve root

[e] LVG - left vagus ganglion

TABLE 7   DETAILED DESCRIPTION OF VARIABLE RESTRICTION ENDONUCLEASE (RE)
SITES IN HSV-1 GANGLION AND TRIGEMINAL NERVE ROOT ISOLATES

Variable RE site[a]	Approximate map location (genome length= 100 map units)	New site within HSV-1 17 RE fragment (Md new fragments)	Novel fragments resulting from fusion of HSV-1 17 fragments (Md)
H1	10		h/o (8.9)
H2	88		m/n (7.4)
K1	2-10	b(6.2/1.8)	
K2	13-17	f(3.4/3.6)	
K3	28-32	m(2.6/0.5)	
K4	28-32	m(1.9/1.2)	
K5	40		p/v (3.9)
K6	59		s/d (10.4)
K7	72-80	g(5.2/1.8)	
B1	14-22	a(7.2/0.8)	
B2	14-22	a(5.8/2.2)	
B3	34-39	g(4.3/0.4)	
B4	44		w/j' (1.7)
B5	45		j'/b' (0.9)
B6	52		d/h (10.3)
B7	74-78	f(2.9/2.1)	

[a] Each site, starting from the left hand end of the HSV-1 17 map is
named by the first letter of the enzyme used and then numbered
consecutively (H, Hind III; K, Kpn I; B, Bam HI)

TABLE 8   VARIABLE RESTRICTION ENDONUCLEASE SITE LOCATIONS OF HSV-1 GANGLION AND TRIGEMINAL NERVE ROOT ISOLATES

Isolate designation
(No. isolates per individual)

		Restriction endonuclease site[a]														
	H1	H2	K1	K2	K3	K4	K5	K6	K7	B1	B2	B3	B4	B5	B6	B7
HSV-1 17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8A-C (3)	-	-	+	+	+	-	-	+	+	-	+	+	-	-	+	+
18A-C (3)	-	-	+	-	-	-	-	+	+	+	-	-	-	+	-	-
20A-C (3)	-	-	+	-	+	-	-	+	+	+	-	-	+	-	-	-
21A-D (4)	+	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-
22A-D (4)	+	-	+	-	-	-	-	+	+	+	-	-	-	-	+	-
23A-C (3)	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	-
24A-F (6)	-	-	+	-	+	-	-	+	+	-	+	-	+	-	-	-
25A-L (12)	+	-	+	-	+	-	-	+	+	-	+	-	+	-	+	-
26A-F (6)	+	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-
27A-H (8)	-	-	+	-	+	-	-	+	+	+	-	-	-	-	-	-
28A-D (4)	-	-	+	-	+	-	-	+	+	-	-	-	-	-	-	-
30A-C (3)	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+	-
31A1	-	-	+	-	+	-	-	+	+	+	+	-	-	-	-	-
31A3	-	-	+	-	+	-	-	+	+	+	-	-	+	+	+	-
31C1 (13)	-	-	+	-	+	-	-	+	+	-	-	-	-	-	-	-
31C2	-	-	+	-	-	-	-	+	+	-	+	-	-	+	+	-
31C3	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-
31C4	-	-	+	-	-	-	-	+	+	+	+	-	-	-	+	-
31G4	-	-	+	-	+	-	-	+	+	+	-	-	-	-	+	-
31K2	-	-	+	-	+	-	-	+	+	-	+	-	-	-	+	-
36A-H (8)	-	-	+	-	+	-	-	+	+	-	+	-	-	-	+	-
37A-J (10)	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-
38A-D (4)	-	-	+	-	-	+	-	+	+	-	+	-	-	-	-	-
40A-C (3)	+	-	+	-	-	-	-	-	+	+	-	-	+	-	+	-
41A-G (7)	+	-	+	-	+	-	-	+	+	+	-	-	-	-	-	-
43A-D (4)	-	-	+	-	+	-	-	+	+	-	+	-	-	-	-	-
49A (6)	-	-	+	-	-	-	-	+	+	-	+	-	-	-	+	-
49B-F	+	-	+	-	-	-	-	+	+	-	-	-	-	+	+	-

[a] Each variable site is indicated as present (+) or absent (-)

TABLE 9   RESTRICTION ENZYME PATTERNS OF SUBLCONES OF ISOLATES 31 A,C, G, and K[a]

Isolate	Bam HI Patterns						Kpn I patterns	
	I[b]	II[c]	III[d]	IV[e]	V[f]	VI[g]	I[i]	II[j]
31A	A1,A2,A3,A5	A1,A2,A5					A3	A1,A2,A5
31C	C1		C4	C2		C3,C5	C1,C3,C5	C2,C4
31G	G1,G3,G5		G2		G4		G4	G1,G2,G3,G5
31K	K5[h]	K1		K2,K3,K4,K5[h]			K1,K2,K3	K4,K5[h]

[a] plaque-purified subclones are designated by a number following the nomenclature of the parental isolate.

[b] Bam HI pattern I is characterized by the presence of d/h and b'/j' fusion fragments.

[c] Bam HI pattern II is charaterized by a cleavage site with fragment a [8.0 Megadaltons (Md)] and the appearance of 2 new fragments of 7.2 Md and 0.8 Md.

[d] Bam HI pattern III is characterized by a cleavage site within fragment a (8.0 Md) and the appearance of 2 new fragments of 5.8 Md and 2.2 Md.

[e] Bam HI pattern IV resembles that of pattern III and also contains a d/h fusion fragment.

[f] Bam HI pattern V resembles that of pattern II and also contains a d/h fusion fragment.

[g] Bam HI pattern VI is identical to that of HSV-1 17.

[h] Subclone K5 has mixed profile of Bam HI patterns II and IV.

[i] Kpn I pattern I is characterized by a cleavage site within fragment m(3.1 Md) and the appearance of 2 new fragments of 2.6 Md and 0.5 Md.

[j] Kpn I pattern II lacks a cleavage site within fragment m.
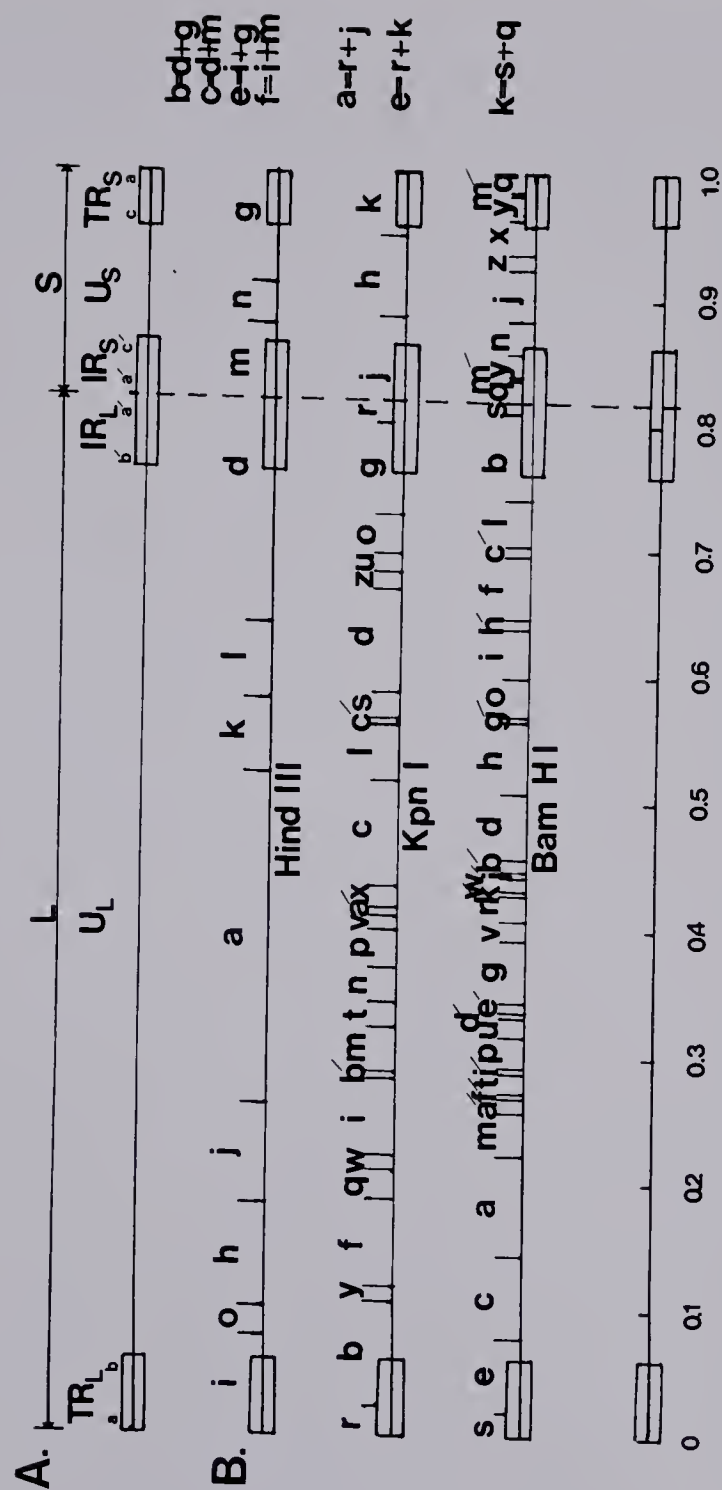
Figure 4

A) HSV genome organization and B) Physical maps for the HSV-1 17 DNA
fragments generated by Hind III, Kpn and Bam HI restriction enzymes.

Figure 5

Autoradiographs of A) Kpn I and B) Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17, HSV-2 HG52 and ganglion isolates 8A-C (●) Novel fusion or cleavage products.
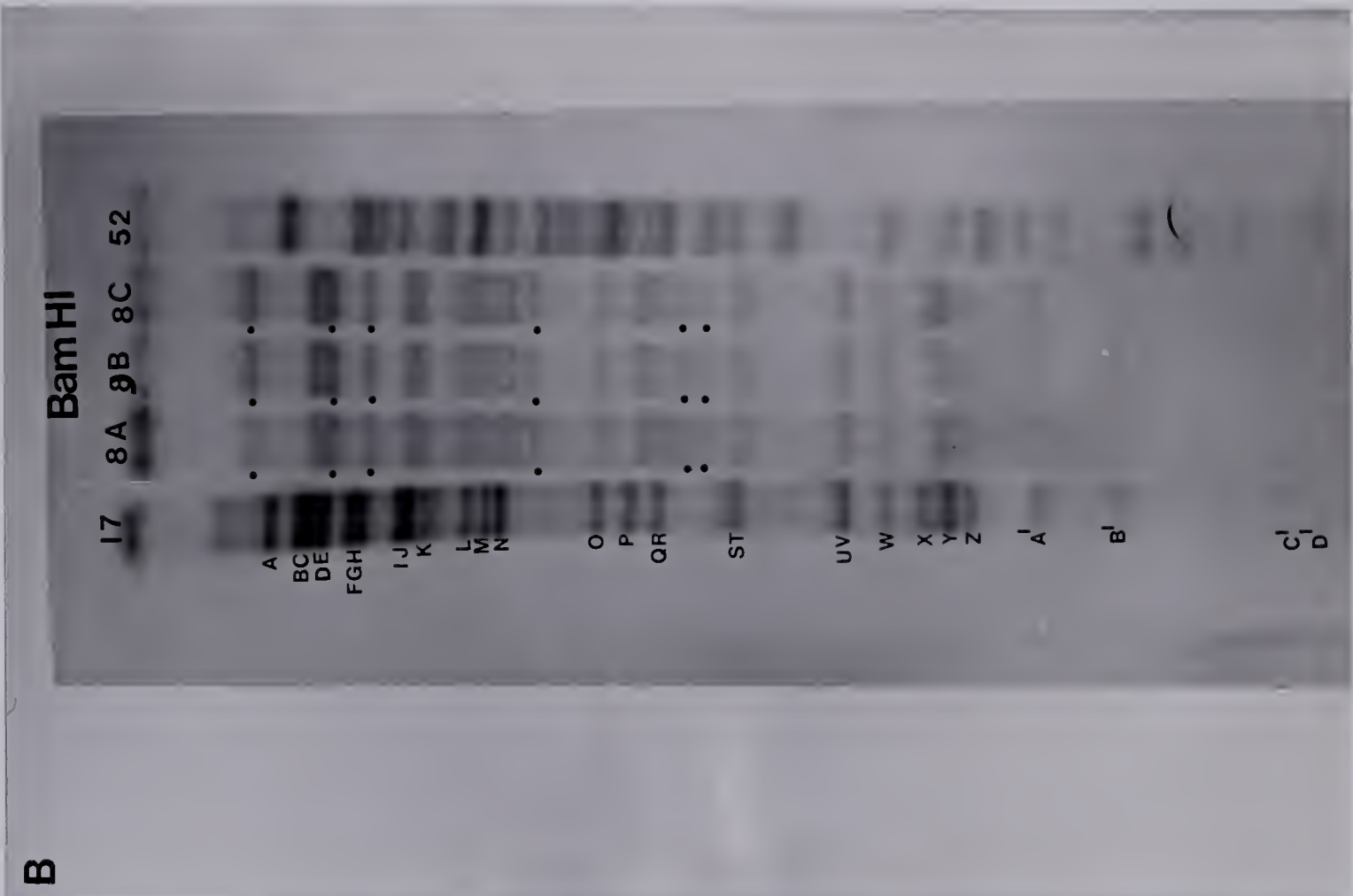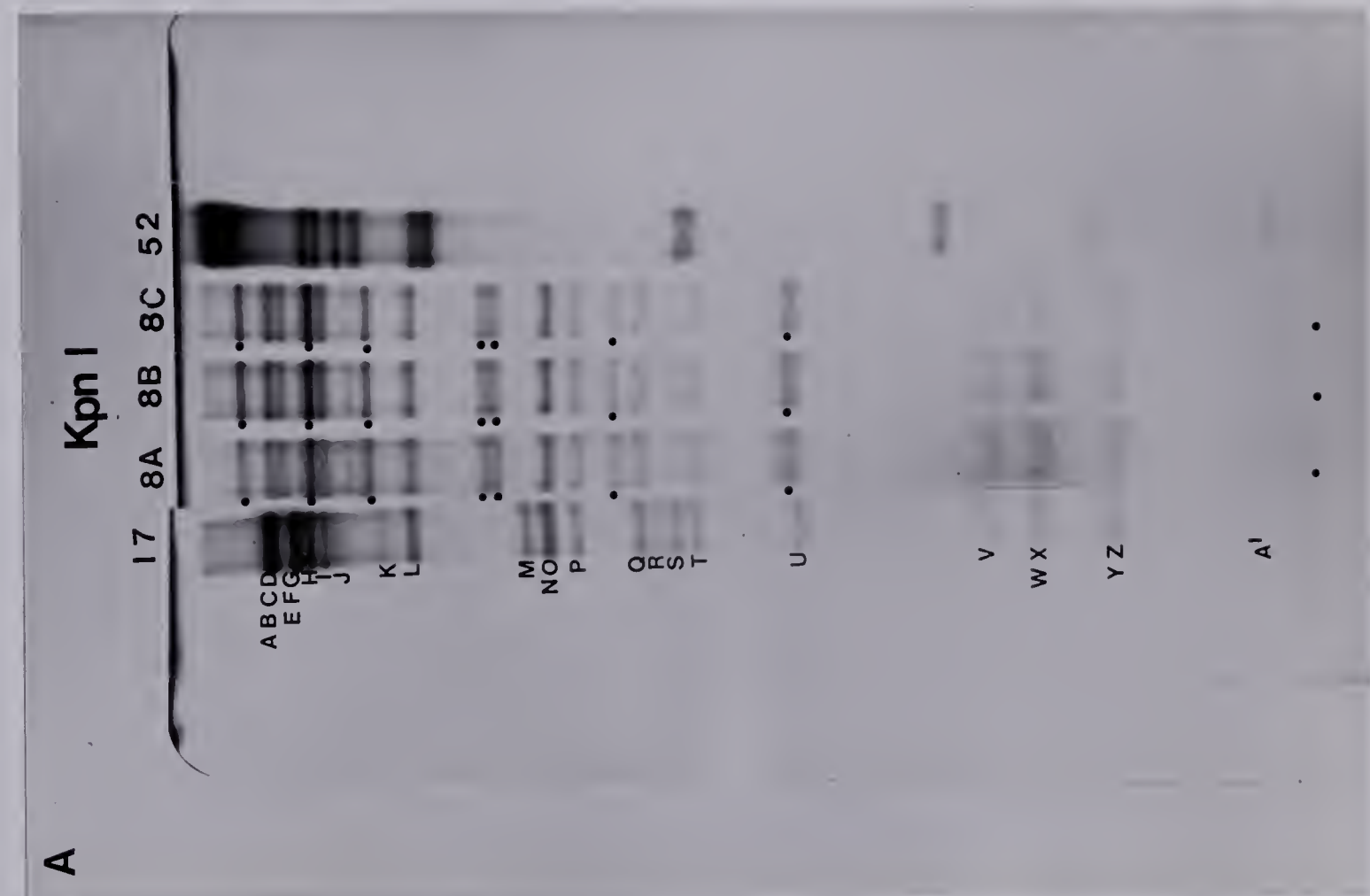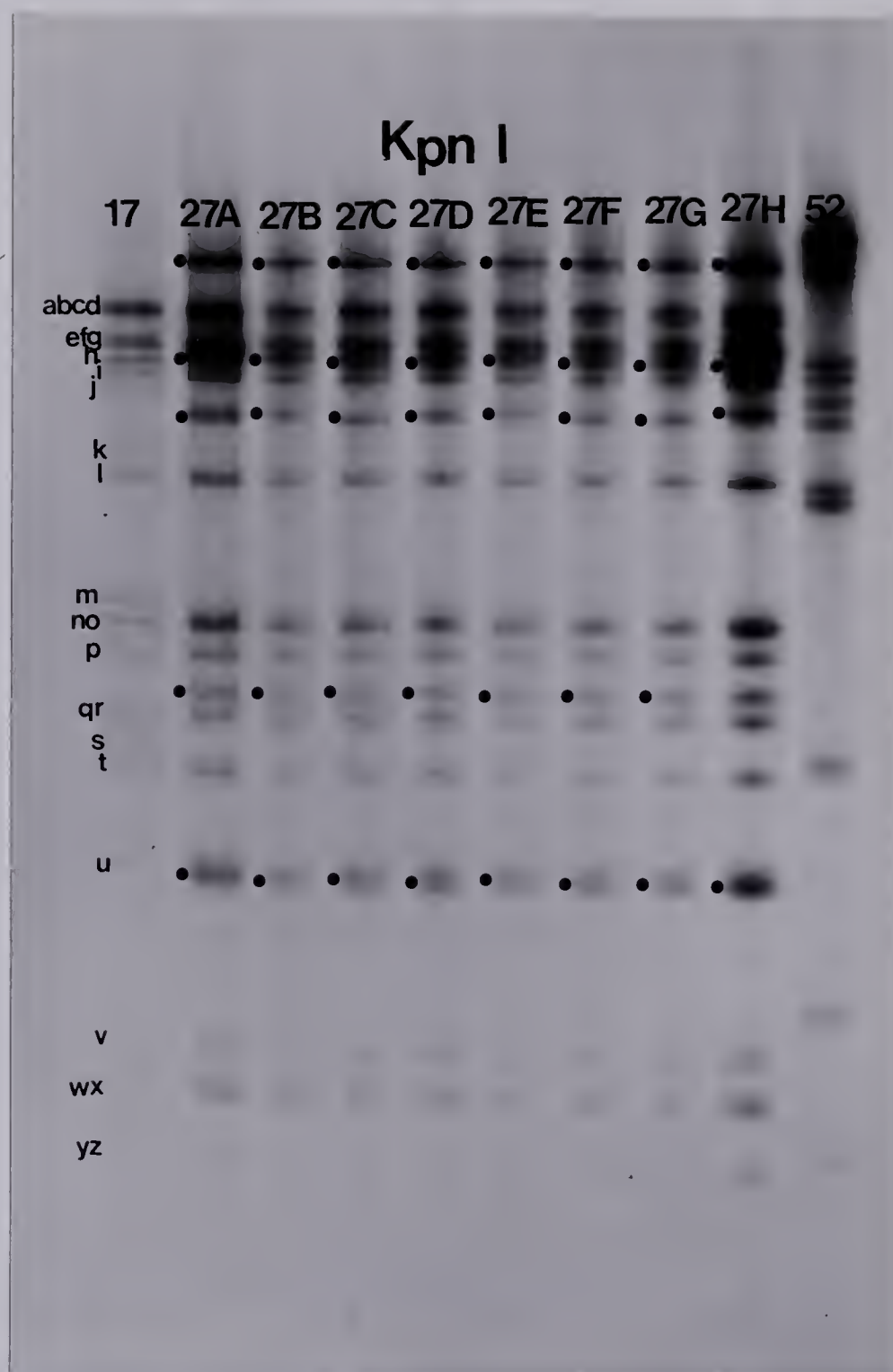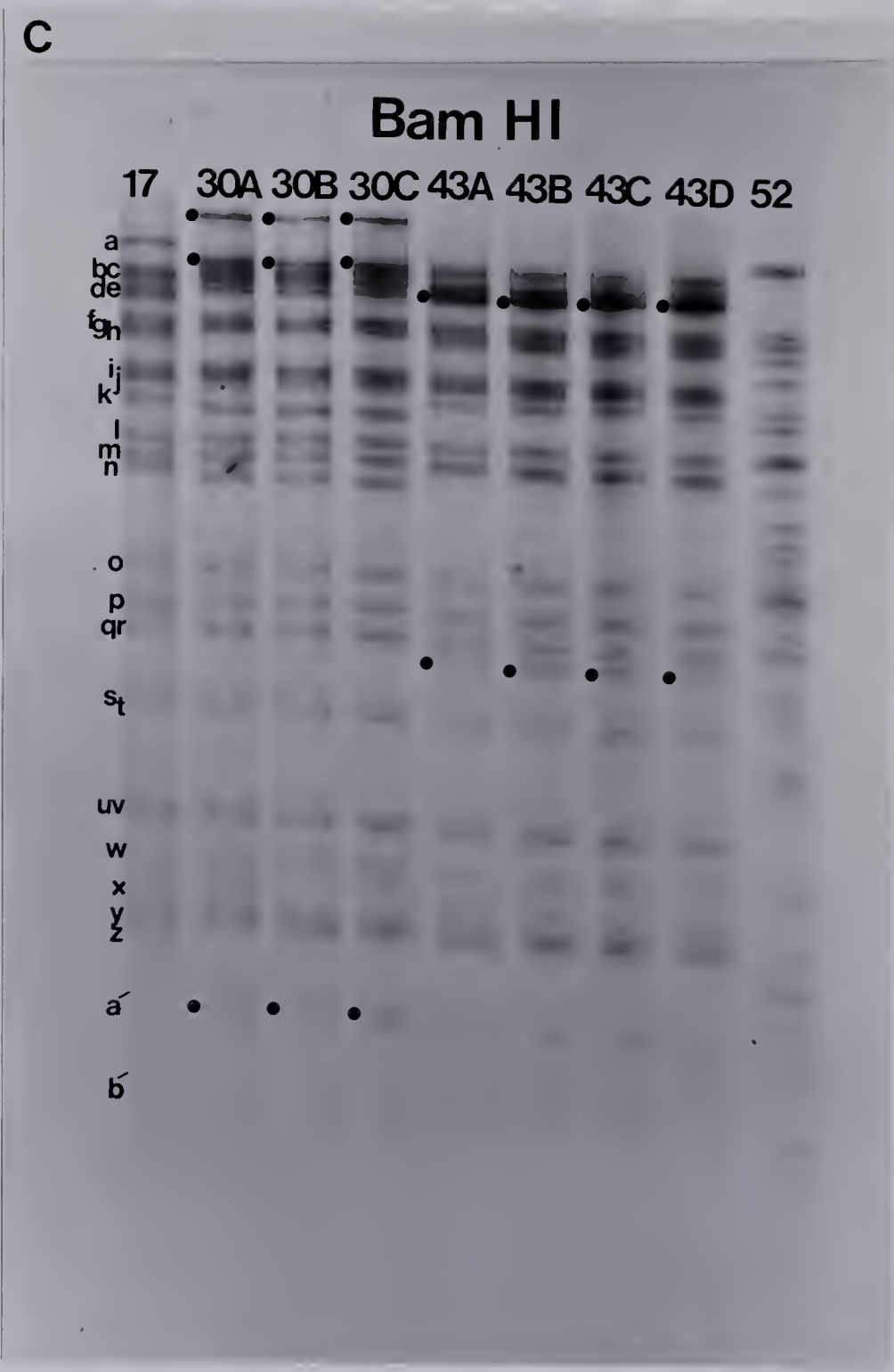
Figure 6

Autoradiographs of A) Hind III B) Kpn I and C) Bam HI digests of
^{32}P-labeled DNA of prototype HSV-1 17 and ganglion isolates 18A-C, 21A-D
and 22A-D (●) Novel fusion or cleavage products.
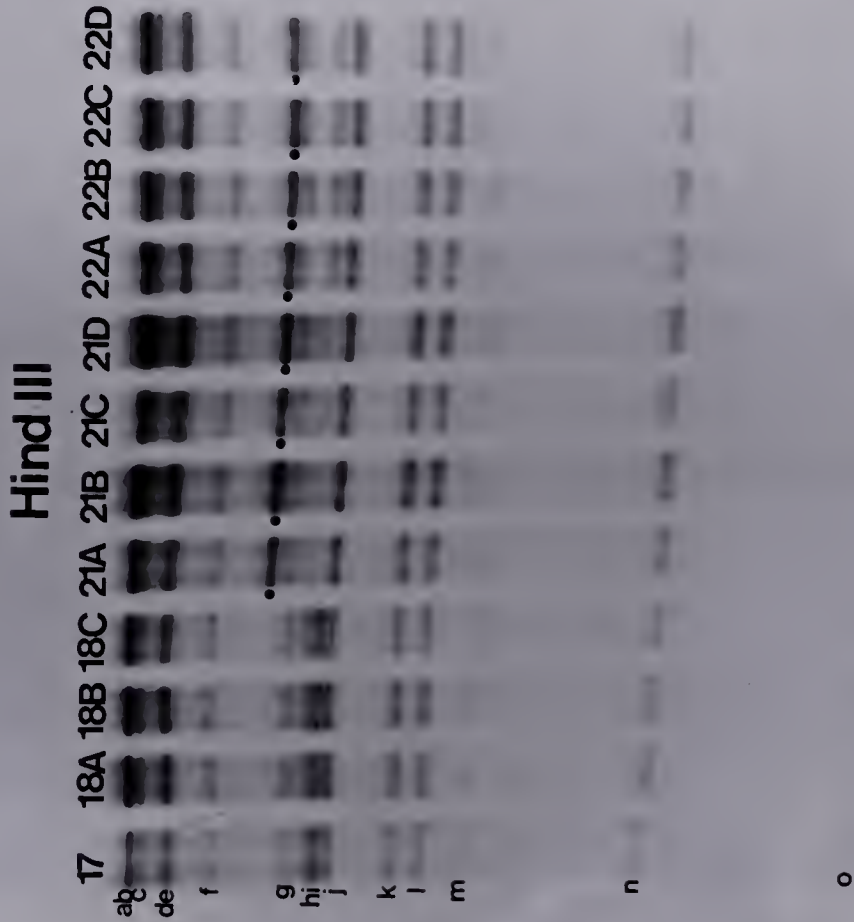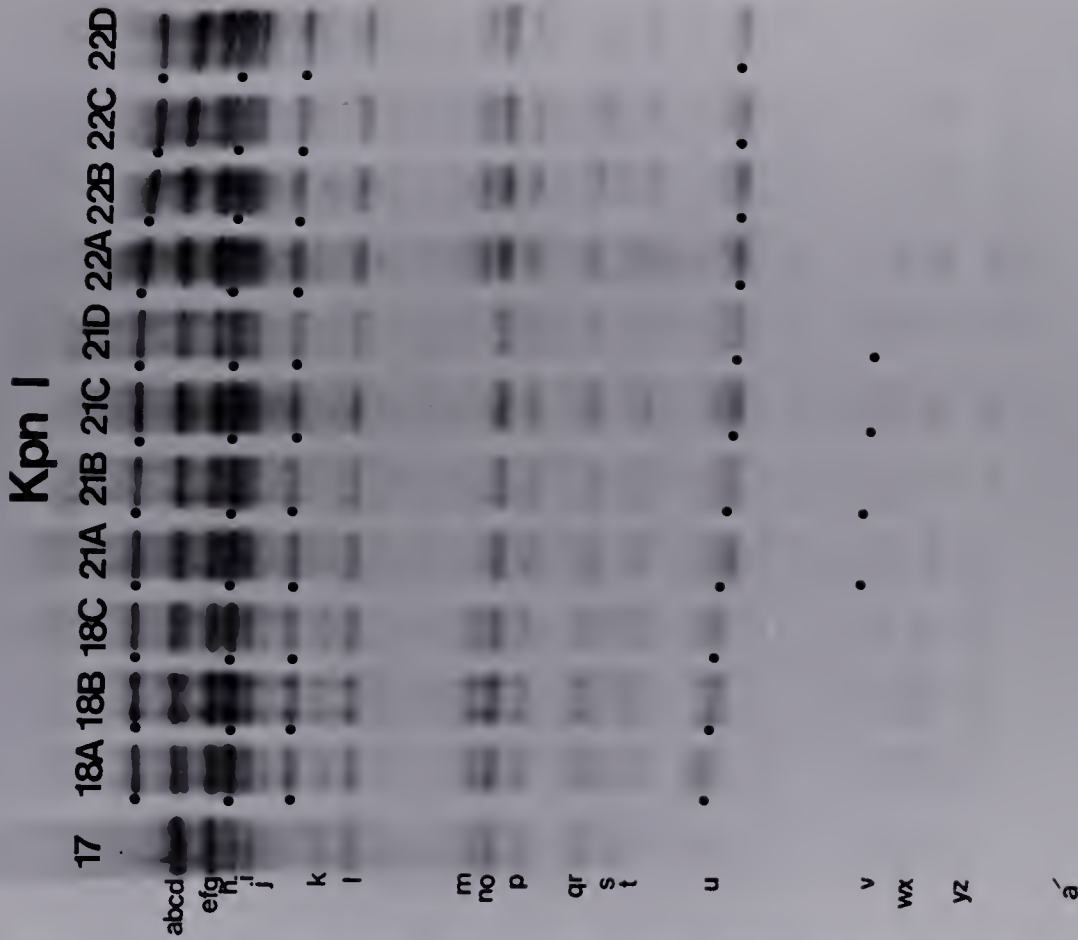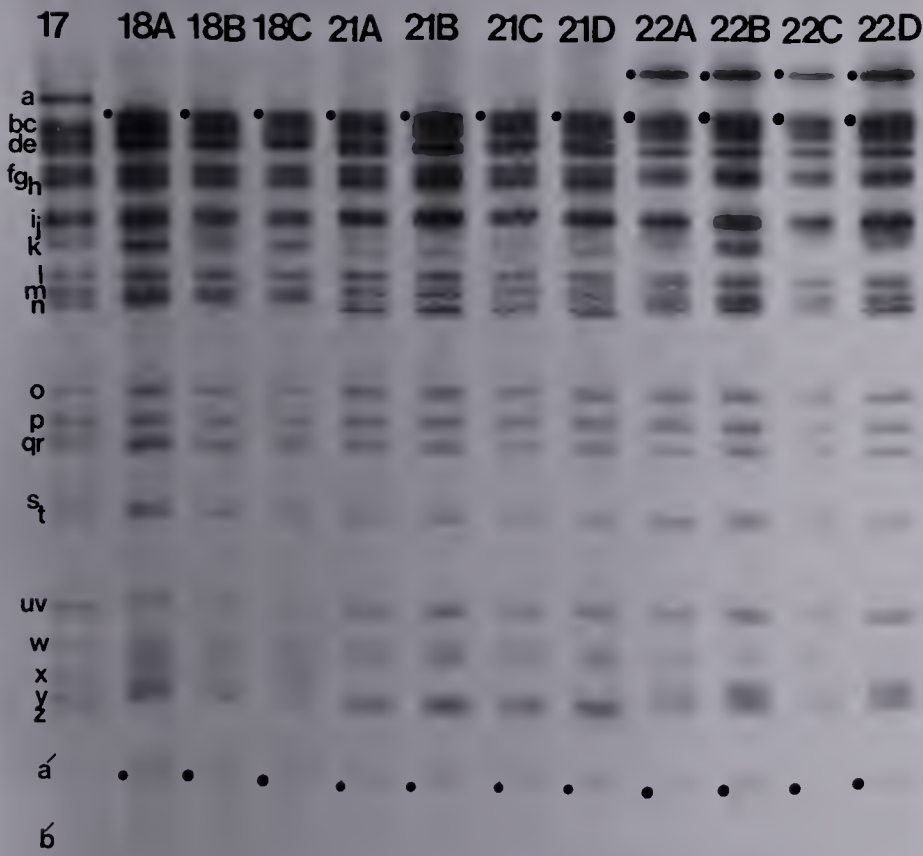
Figure 7

Autoradiographs of A) Hind III B) Kpn I and C) Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17 and HSV-2 HG52 and ganglion isolates 23A-C (●) Novel fusion or cleavage products.

Kpn I

17 23A 23B 23C 52

abcd efg h i j k l m no p qr s t u v wx yz a'

Hind III

17 23A 23B 23C 52

ab c de f g hij j k l m n o
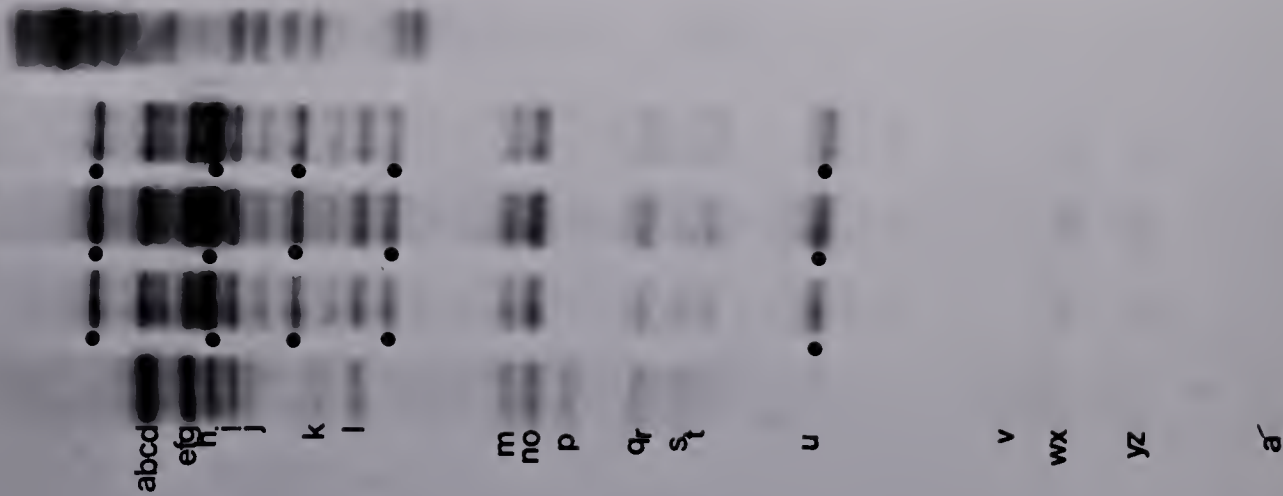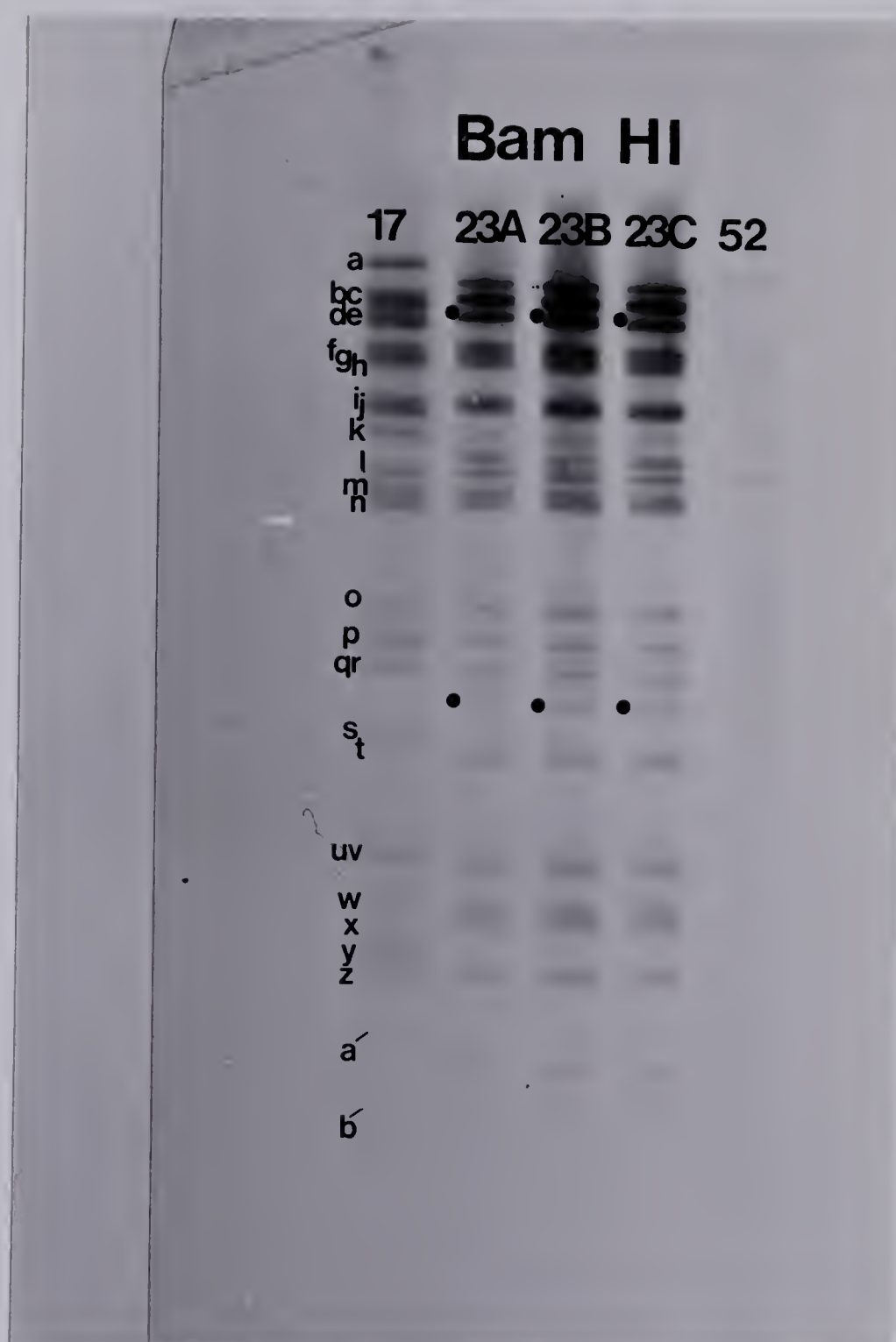
Bam HI

Figure 8

Autoradiograph of Kpn I digests of ^{32}P-labeled DNA of prototypes HSV-1
17 and HSV-2 HG52 and ganglion isolates 24A-F. (●) Novel fusion or
cleavage products.

Figure 9

Autoradiograph of Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1
17 and HSV-2 HG52 and ganglion isolates) 25A-F and B) 25G-L. (●) Novel
fusion or cleavage products.
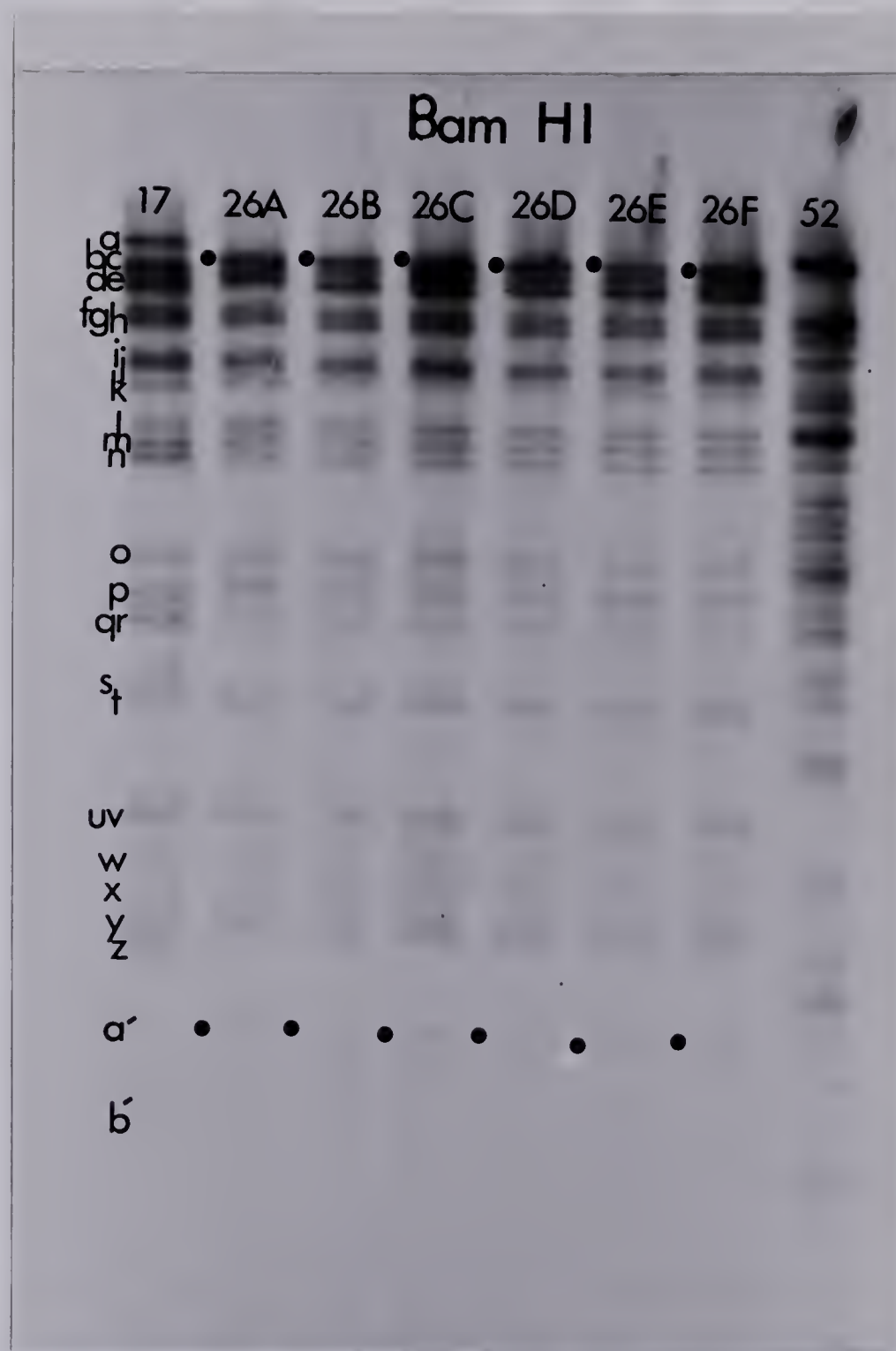
Figure 10

Autoradiograph of Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17 and HSV-2 HG52 and ganglion isolates 26A-F (●) Novel fusion or cleavage products.

Figure 11

Autoradiograph of Kpn I digests of ^{32}P-labeled DNA of prototypes HSV-1 17 and HSV-2 HG52 and ganglion isolates 27A-H (●) Novel fusion or cleavage products.

Figure 12

Autoradiographs of A) Hind III B) Kpn I and C) Bam HI digests of
^{32}P-labeled DNA of prototypes HSV-1 17 and HSV-2 HG52 and ganglion
isolates 30A-C and 43A-D (●) Novel fusion or cleavage products.

A   Hind III

B   Kpn I

Bam HI

Figure 13

Autoradiographs of A) and B) Kpn I and C) Bam HI digests of ^{32}P-labeled
DNA of prototypes HSV-1 17 and HSV-2 HG52 and ganglion and trigeminal
nerve root isolates 31A-M.
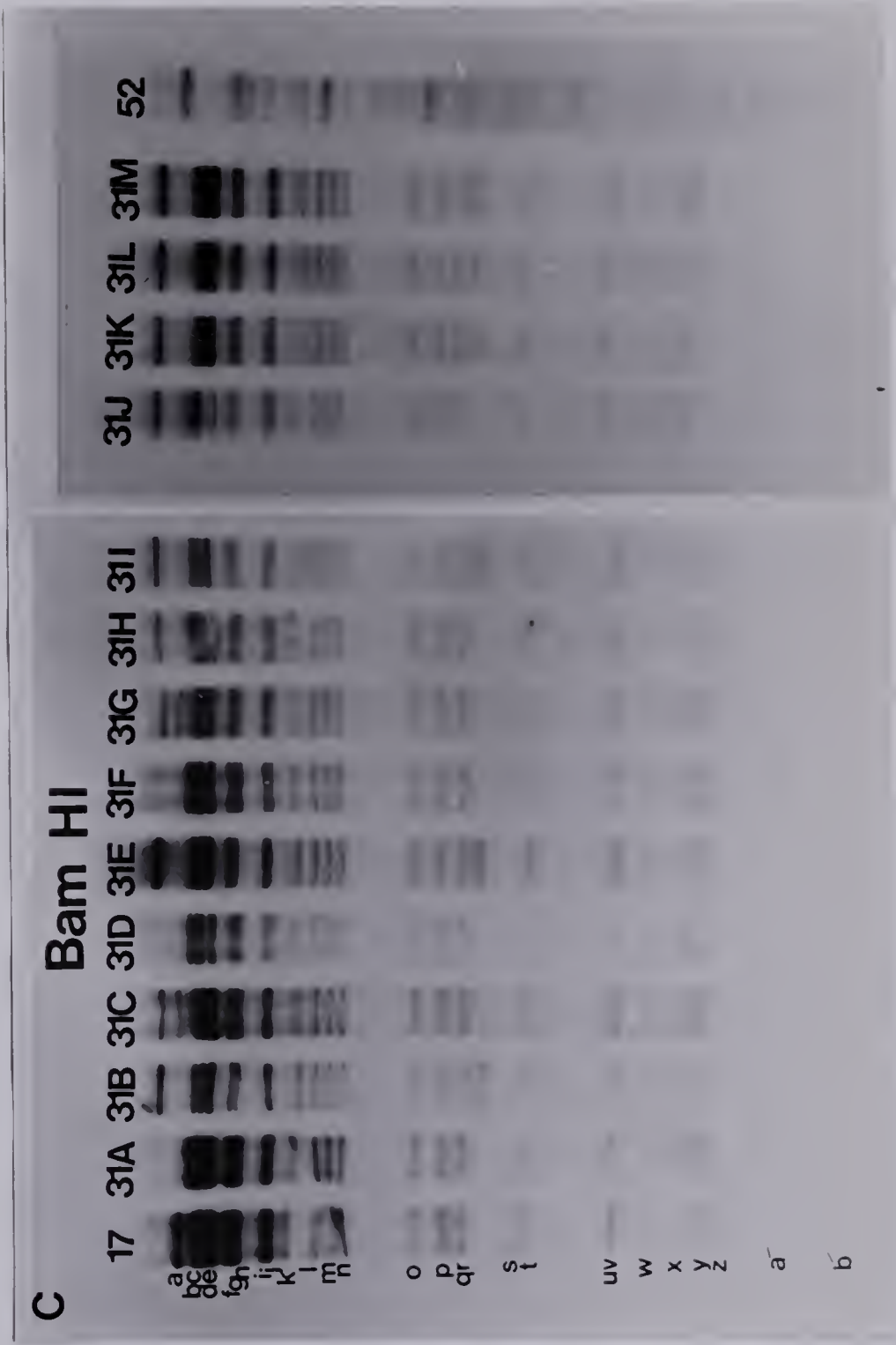
Figure 14

Autoradiographs of digests of ^{32}P-labeled DNA of HSV-1 17, HSV-2 HG52, and various subclones of isolates 31A,C,G and K.

A. Kpn I profiles of representative subclones G2 and G4 derived from isolate 31G. Strain G4 has a Kpn pattern I profile whereas strain G2 has a Kpn pattern II profile (see Table 12).

B. Bam HI profiles of prototypes subclones 31 A3, C3, K1, K2, G2 and G4. Strain A3 has a Bam HI pattern II profile, K1 has a Bam HI pattern I profile, and K2 has a Bam HI pattern IV profile. The Bam HI profile of strain C3 is identical to that of strain 17 (pattern VI). Strain G2 has a Bam HI pattern III profile and G4 has a Bam HI pattern V profile (Table 12). (●) Novel fusion or cleavage products.
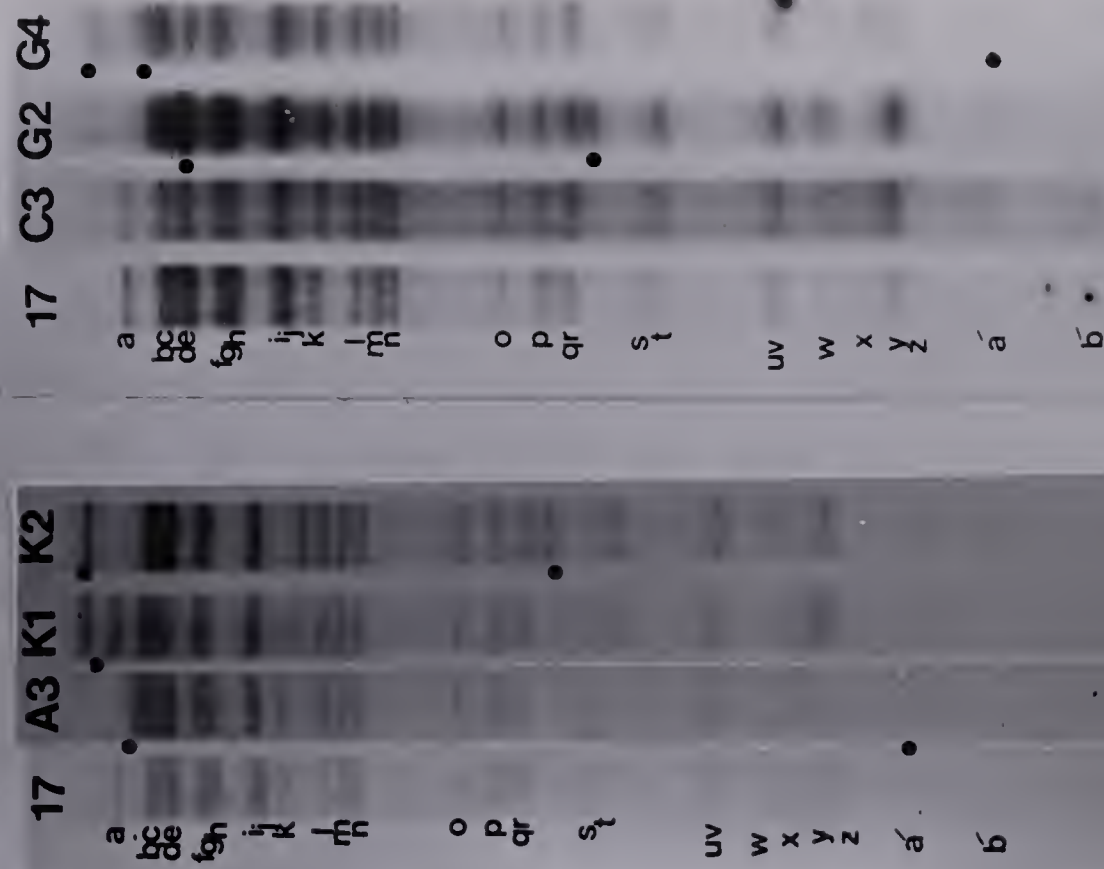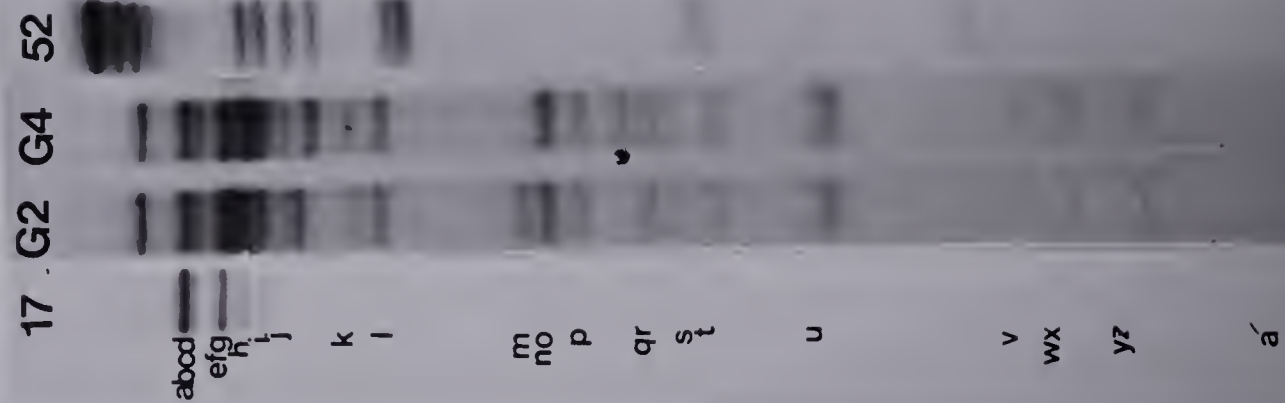
Figure 15

Autoradiographs of digests of ^{32}P-labeled DNA of HSV-1 17, and subclones
of isolates 31A,K,C, and G run on 3.5% polyacrylamide gels.

A. Kpn I profiles. The 0.5Md cleavage product of Kpn I-m which
migrates between fragments a' and b' is shown.

B. Bam HI profiles. The absence of fragments b' and j' indicating the
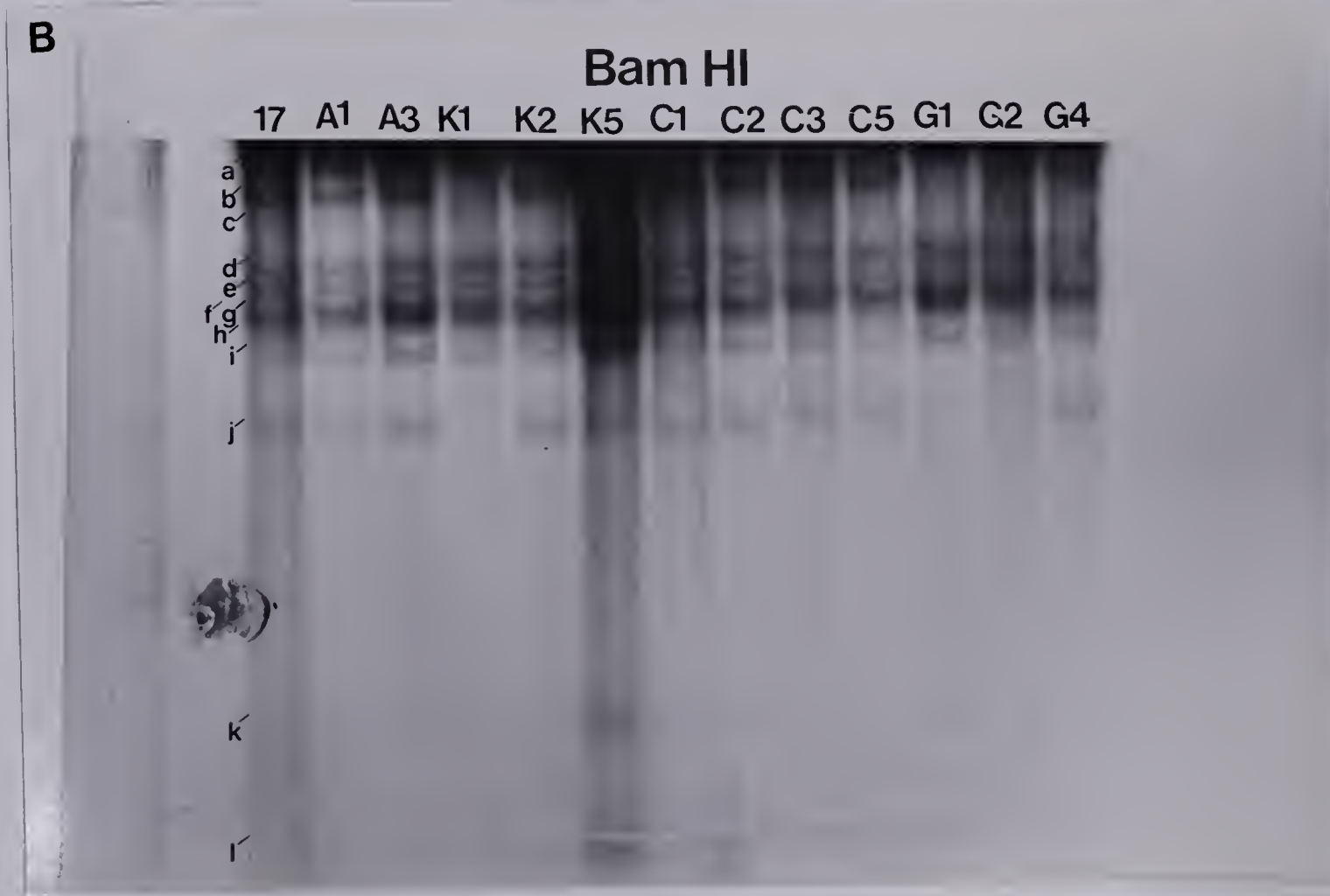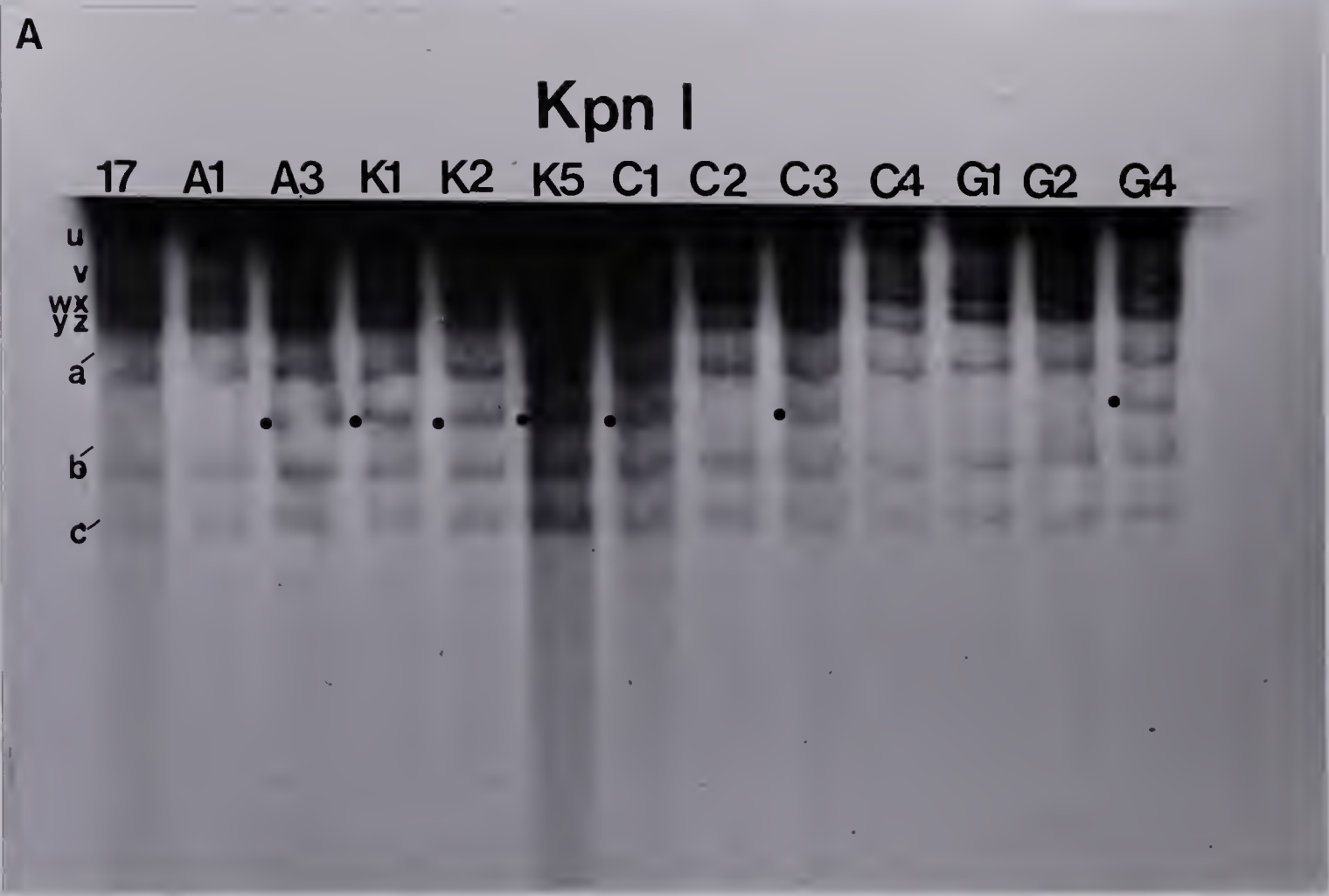loss of a Bam III restriction site is demonstrated.

A. Kpn I

17 A1 A3 K1 K2 K5 C1 C2 C3 C4 G1 G2 G4

B. Bam HI

17 A1 A3 K1 K2 K5 C1 C2 C3 C5 G1 G2 G4

Figure 16

Hybridization of ^{32}P-labeled DNA fragments of HSV-1 strain KOS to the DNAs of HSV-17 and subclones of isolates 31C, G and K. HSV-1 17 fragments are labeled according to standard nomenclature.

A. Bam HI digests of subclones K1, K5, C1, C2, C3, C4, G1, G2 and G4 hybridized to HSV-1 KOS BglII-k

B. Bam HI digests of subclones K1, K5, C1, C2, C3, C4 and G1 hybridized to HSV-1 KOS BglII-j1.

C. Kpn I digests of subclones C1, C2, C3, C4, and G1 hybridized to HSV-1 KOS BglII-j1.
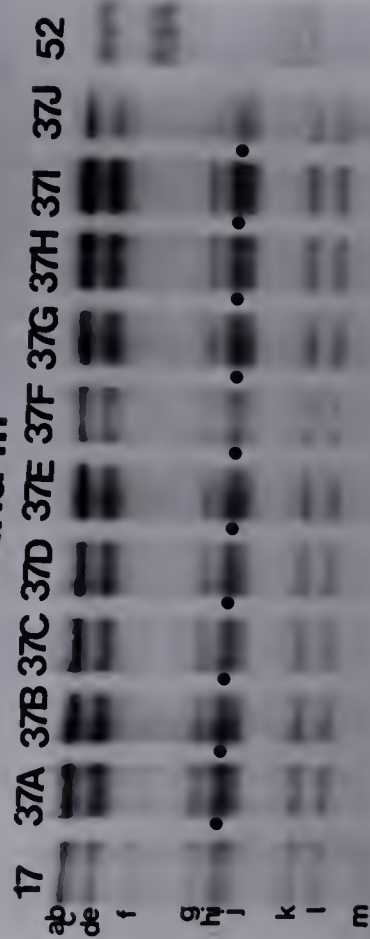
Figure 17

Autoradiograph of Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17, HSV-2 HG52 and ganglion isolates 36A-H (●) Novel fusion or cleavage products.

Figure 18

Autoradiographs of A) Hind III B) Kpn I and C) Bam HI digests of
^{32}P-labeled DNA of prototypes HSV-1 17, HSV-2 HG52 and ganglion isolates
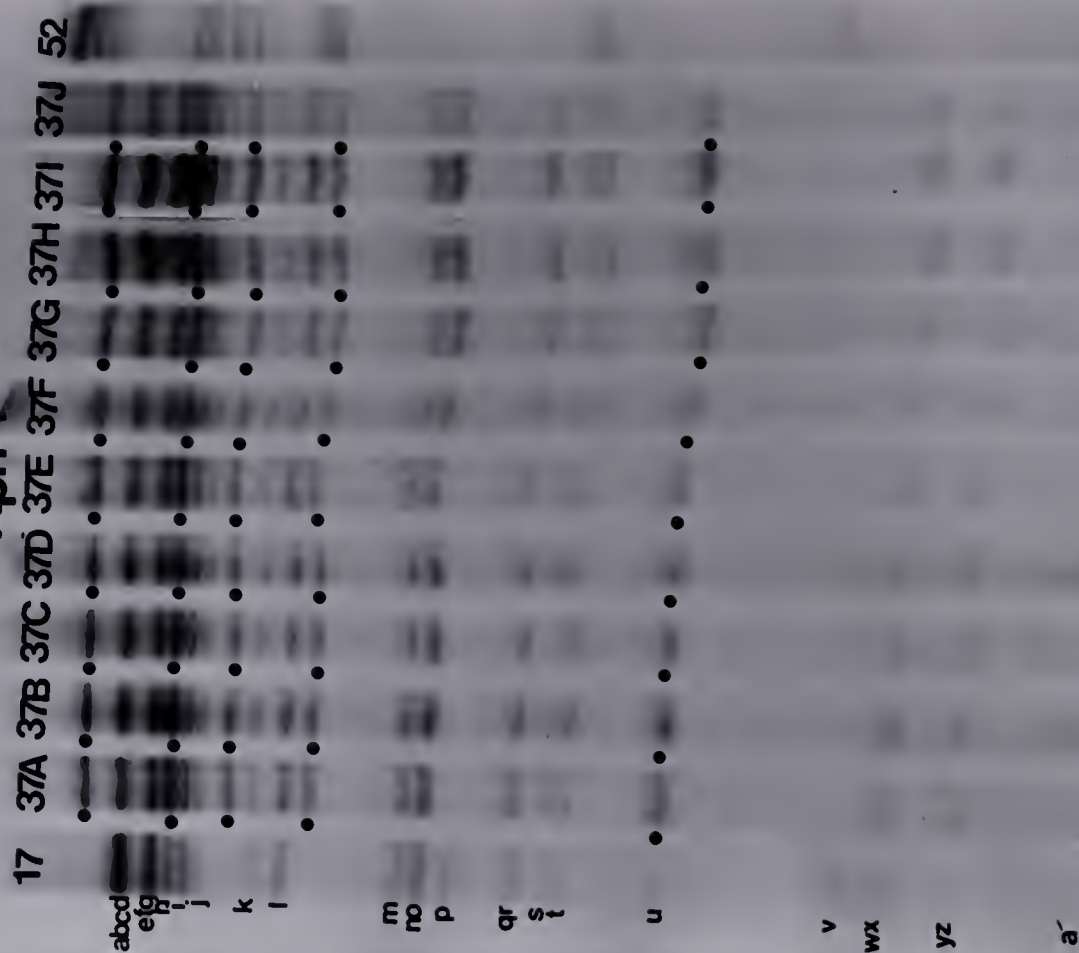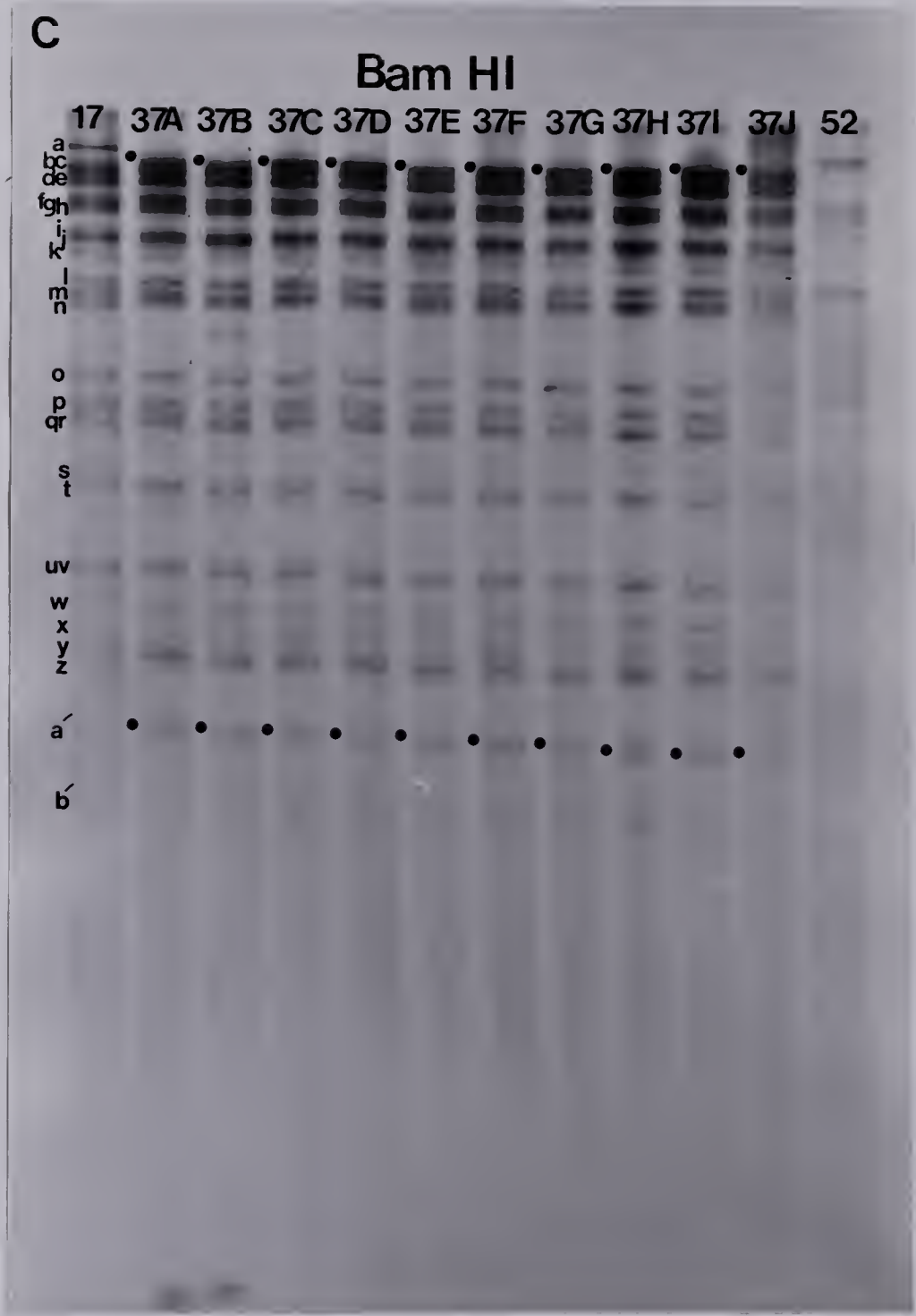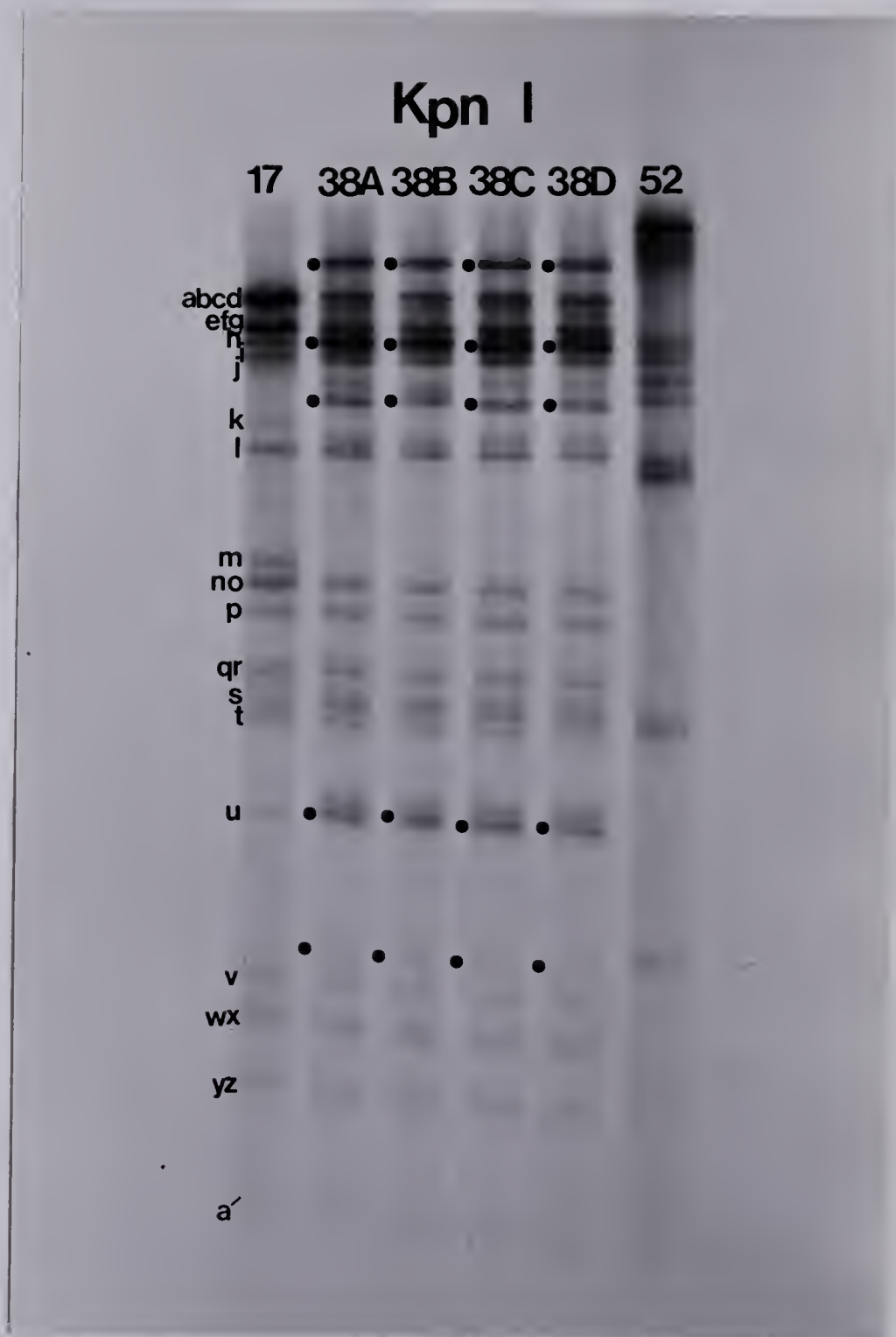37A-J (●) Novel fusion or cleavage products.

Figure 19

Autoradiograph of Kpn I digests of ^{32}P-labeled DNA of prototypes HSV-1 17, HSV-2 HG52 and ganglion isolates 38A-D (●) Novel fusion or cleavage products.

Figure 20

Autoradiographs of A) Kpn I and B) Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17, HSV-2 HG52 and ganglion isolates 40A-C (●) Novel fusion or cleavage products.
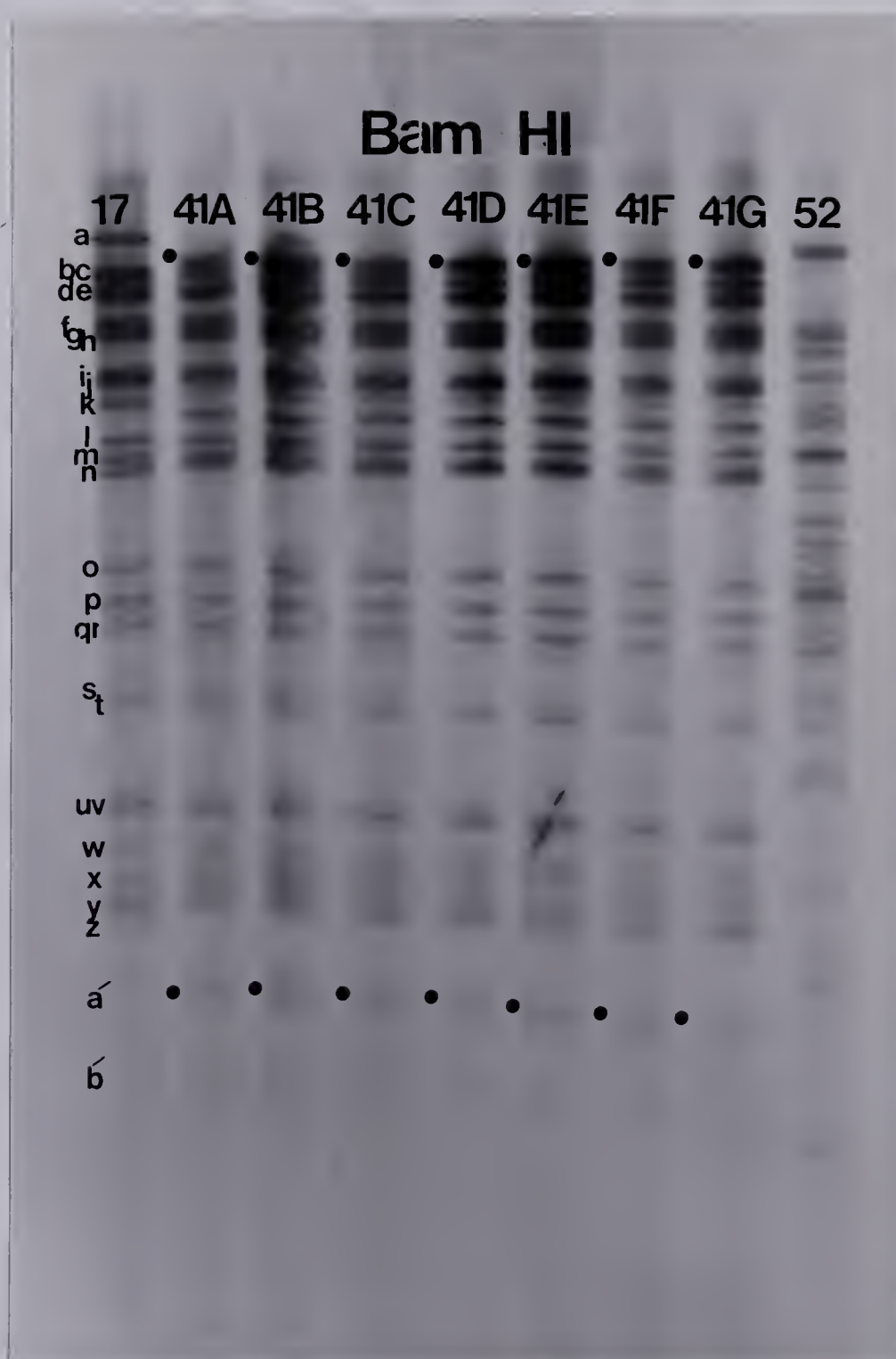
Figure 21

Autoradiograph of Bam HI digests of ^{32}P-labeled DNA of prototypes HSV-1 17, HSV-2 HG52 and ganglion isolates 41A-G (●) Novel fusion or cleavage products.

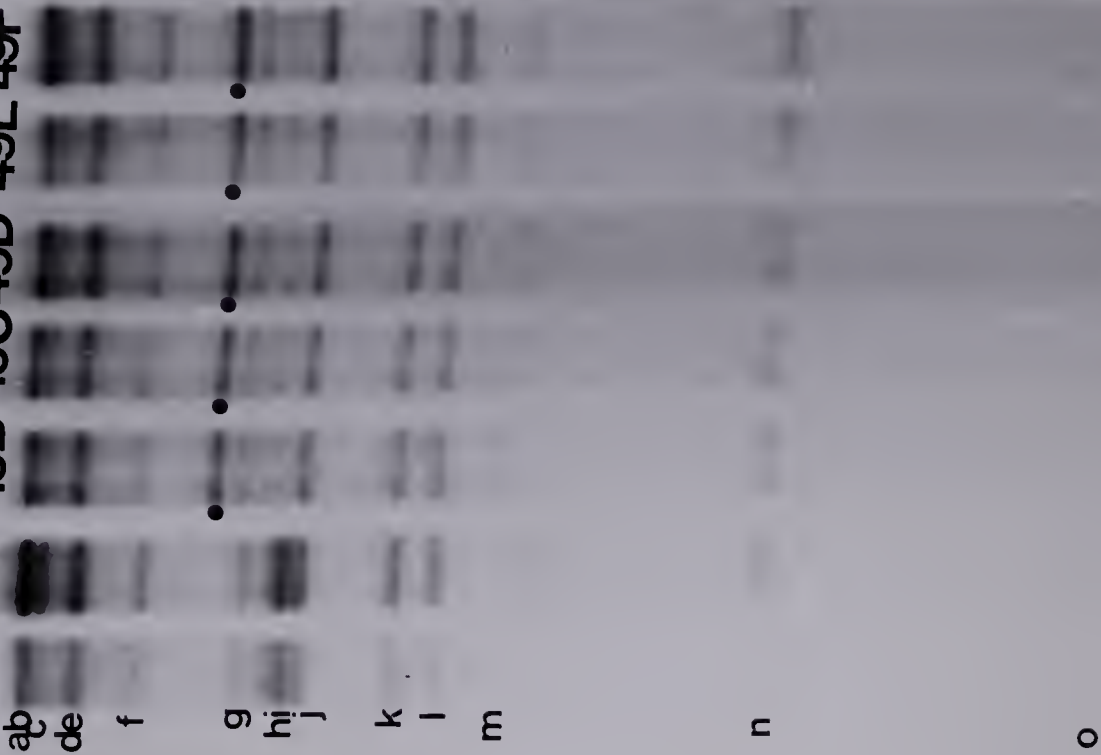Figure 22

Autoradiographs of A) Hind III B) Kpn I and C) Bam HI digests of
^{32}P-labeled DNA of prototpyes HSV-1 17, and ganglion isolates 49A-F (●)
Novel fusion or cleavage products.

C

Bam HI

17 49A 49B 49C 49D 49E 49F
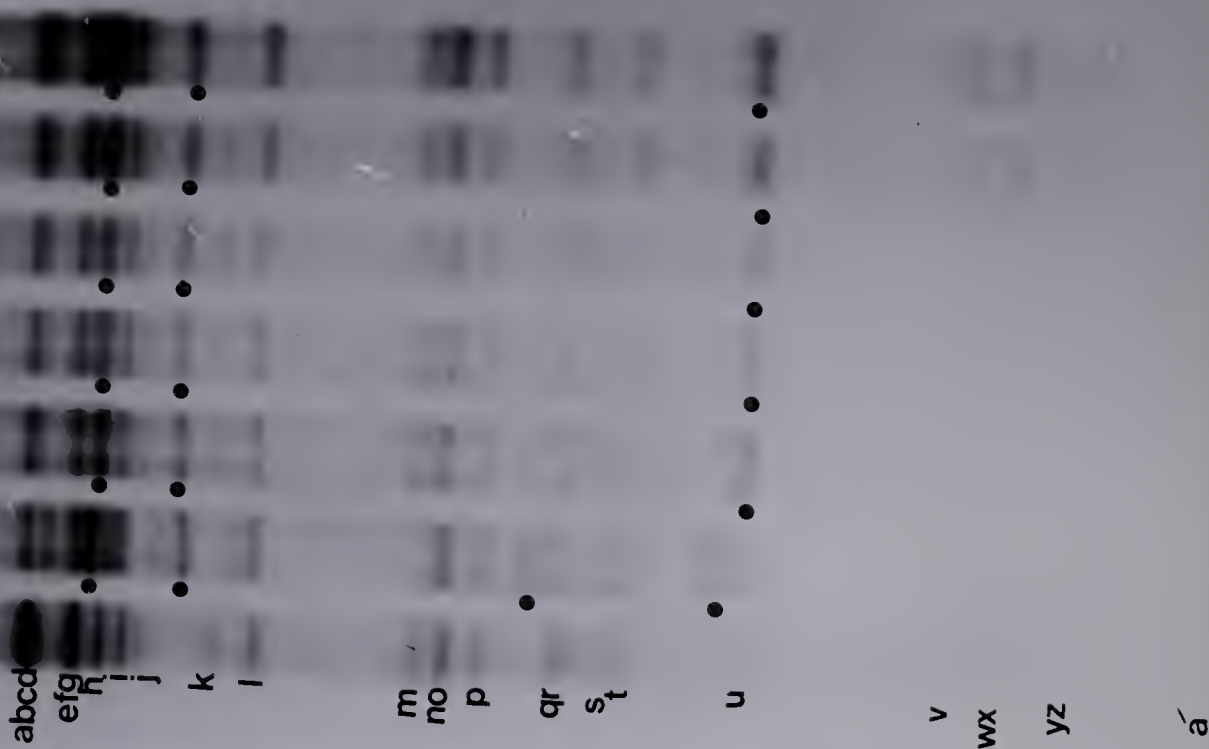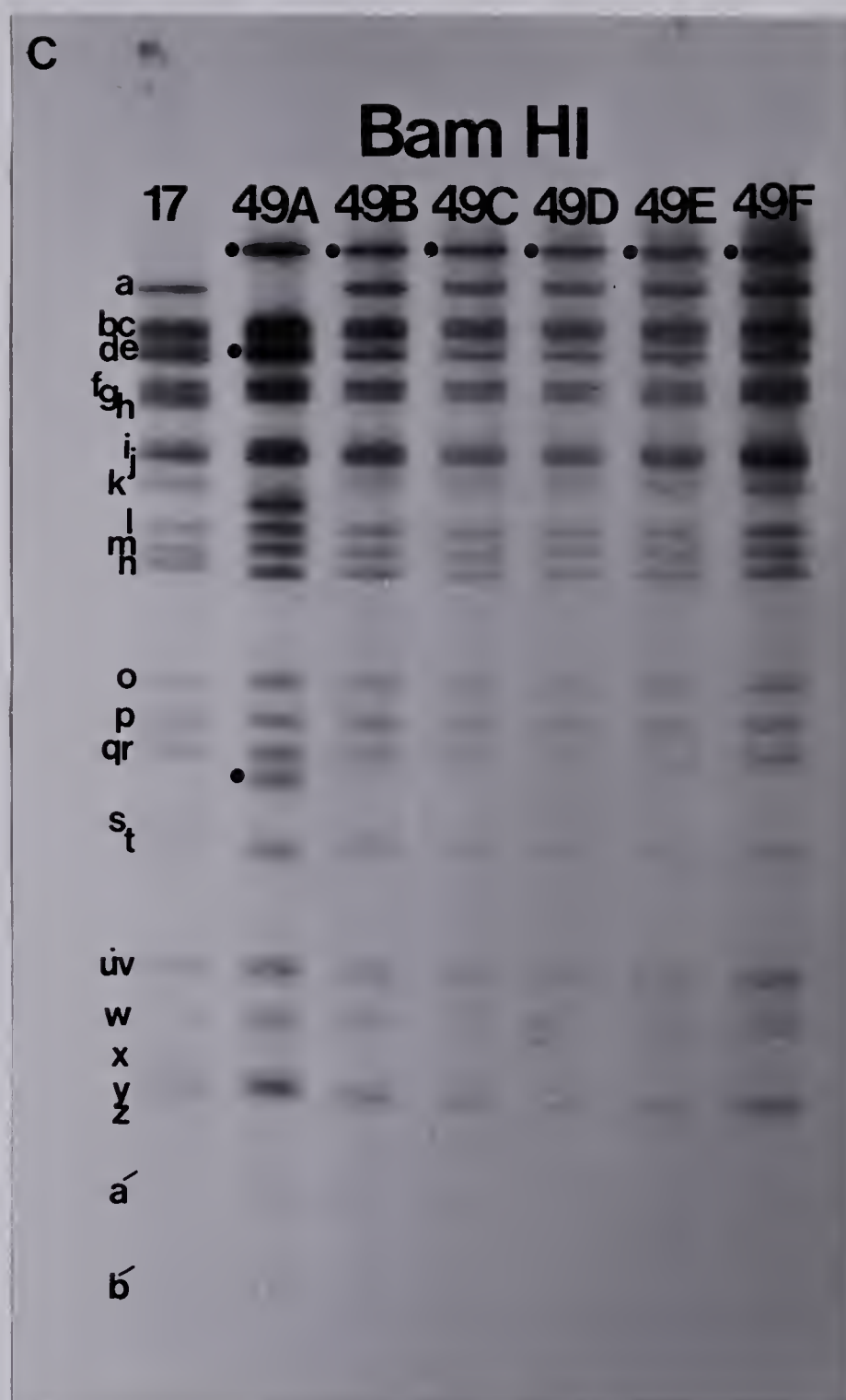
Figure 23

Summary of map locations of variable restriction endonuclease cleavage
sites for Hind III, Kpn I and Bam HI. The physical map locations for
HSV-1 17 are given. Filled inverted triangles point to restriction
endonuclease sites absent in the DNAs of some human HSV-1 strains. Open
inverted triangles point to restriction endonuclease sites present in
the DNAs of some human HSV-1 strains but absent in HSV-1 17 DNA.
Precise locations of novel cleavage sites have not been determined. The
nomenclature of sites is described in Table 10.

b+d+g
c+d+m
e+i+g
f+i+m

i   o   h   j   a   k   l   d   m   n   g

Hind III

H1   H2

a=r+j
e=r+k

r   b   y   f   qw i   bmtnp yax   c   l cs d zuo   g r j   h   k

Kpn I

K1   K2   K3K4   K5   K6   K7

k=s+q

s   e   c   a   maftpue g vr b d h go ihf cl b sdyn j z x y m
                                                           l   q

Bam HI

B1 B2   B3   B4B5   B6   B7
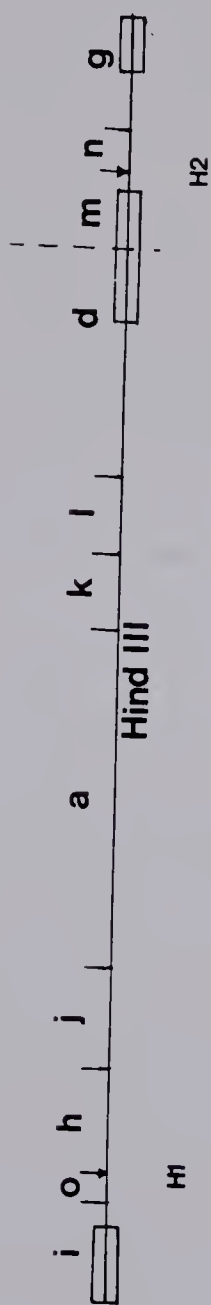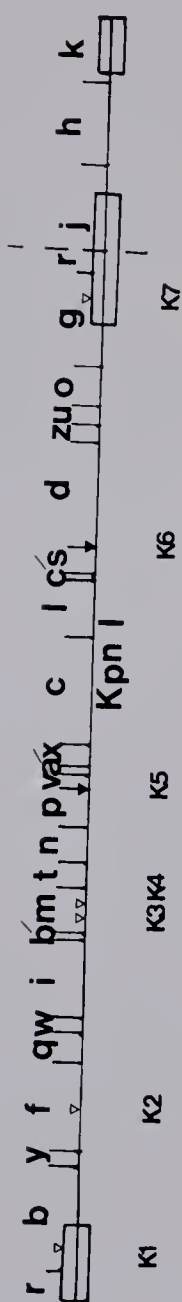
CHAPTER VII

CONCLUDING REMARKS

Though the presence of persistent HSV in human neurological tissue was suggested around the turn of this century, it was not until the early 1970's that latent HSV was recovered from hu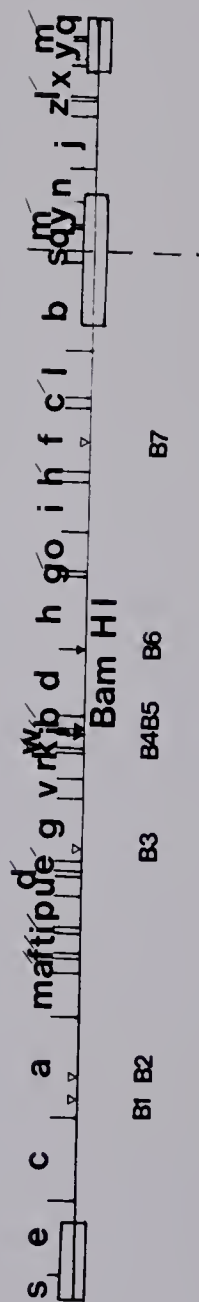man ganglia. In the decade since, many important questions regarding the mechanisms whereby the latent state is established, maintained and reversed to evince overt disease remain to be answered.

The above studies have focused on the biological and biochemical properties of latent HSV present in the ganglia and associated structures derived from unselected human cadavers. Parameters have been identified which optimize recovery of latent virus from human cells cultured in vitro, but additional manipulations which may enhance this process can be imagined. The presence in culture media of nerve growth factor, neuroactive transmitters, cyclic nucleotides, prostaglandins or tumor promoters may also effect in vitro reactivation. The use of other virus activating agents such as UV-irradiation, BudR or temperature manipulations may also prove fruitful.

The present study documents recovery of latent HSV from trigeminal nerve roots and suggests latent virus is not restricted to the autonomic and sensory ganglia of humans. Interest in additional sites of latent or possibly defective genomes stems from the known association of HSV with an acute encephalitis and putative involvement of HSV genes in other clinical diseases such as cervical carcinoma and possibly chronic degenerative diseases of the CNS. The idea has also been raised that cell types other than ganglionic neurons can be a source of latent and reactivable HSV. Though the majority of studies addressing the question

of cell type involved in latent interactions indicate the neuron is the predominant cell type harboring HSV, this association may not be exclusive. The application of cell identification techniques to human cell cultures which subsequently release HSV is the obvious approach needed to clarify this issue. The use of such techniques, combined with the identification of viral nucleic acids by in situ hybridization and viral proteins by immunofluorecence will also aid in a more precise association between the presence of latent virus and specific human cells.

The above studies have indicated that in a small number of cultures negative for spontaneous virus release, virus specific information can be detected by the use of genetic probes in the form of heterotypic HSV ts mutants. However, the low efficiency of this technique makes it obvious that more sensitive methods must be employed to detect viral genomes in very low copy numbers. In situ nucleic acid hybridization assays appear to have the most potential in terms of identifying occasional cells which harbor latent virus. Additional experiments utilizing this technique on human tissue are therefore warranted but must consider recent findings that portions of the HSV genome share homology with mammalian cell DNA.

The technique of in vitro reactivation of latent HSV has also been applied to the study of agents able to inhibit expression of latent genomes. The continuous presence of acyclovir prevented virus reactivation in all cultures tested. Similar experiments have been conducted by others to test the efficacy of various drugs and agents able to block the reactivation process. However, it must be realized that the inhibition of virus reactivation is not necessarily equivalent

to the arrest of viral replication once reactivation has occurred. Until the precise nature of reactivation is defined in molecular terms the application of viral inhibitors towards the creation of in vitro model systems of latent infections will be limited. Nevertheless, the use of antivirals such as ACV in inhibition studies described here has great utility in assessing agents which may be administered to human patients to prevent recurrent HSV infections.

The molecular analysis of multiple isolates of latent HSV recovered from individual human hosts has revealed the majority of individuals harbor a unique virus strain within single or multiple neuroanatomical sites. However, the finding that some individuals maintain more than one strain of HSV-1 in latent form is significant in terms of the new questions it raises. This observation invites re-examination of existing theories concerned with establishment of latent infections and the role of the immune response in modulating access of superinfecting HSV strains to ganglia already colonized with latent virus. As several animal studies have indicated, re-inoculation of previously infected animals may fail to establish latent infections if the initial infecting strain is present in high titers. Presumably, the characteristics of initial infection such as dose and route of inoculation, severity of infection and properties of individual strains may determine if an immunocompetent individual will be able to support multiple latent infections.

The ability of humans to harbor multiple strains of HSV in either overt or latent form opens up the possibility of in vivo genetic interactions between different strains. Though in vivo recombination between HSV strains has never been documented in humans, surely such

interactions are feasible. The generation of genetic variants in this way may affect the epidemiology of HSV infections or may contribute to the pathogenesis of rare diseases of the human nervous system whose etiological agents have not yet been identified. What is apparent is that the pathogenesis of HSV infections in humans is variable and not fully understood. Many generalities now held will be tested as new data accumulates.

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APPENDIX: Publications arising from this thesis

Lewis, M.E. and Warren, K.G. 1980. Herpes zoster mandibularis. Can. J. Neurol. Sci. 7(2):153-155.

Lewis, M.E., Warren, K.G., Jeffrey, V. and Shnitka, T.K. 1982. Factors affecting recovery of latent herpes simplex virus from human trigeminal ganglia. Can. J. Microbiol. 28:123-129.

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Lewis, M.E., Leung, W.-C., Jeffrey, V.M. and Warren, K.G. 1983. Detection of multiple strains of latent herpes simplex virus type 1 within individual human hosts (submitted for publication).

Lewis, M.E., Warren, K.G., Leung, W.-C. Comparison by restriction enzyme analysis of the DNAs of latent herpes simplex virus type 1 strains isolated from the trigeminal nerve complex of individual humans. (manuscript in preparation).